Synthetic and Endogenous Cannabinoids Inhibit Breast Cancer Cell Growth and Metastasis

Honors Research Thesis

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by

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Abstract

With one million cases diagnosed yearly worldwide, breast cancer is the second most common cancer in women. Metastasis to the brain is the leading cause of death in breast cancer patients due to the inability of drug treatments to cross the blood brain barrier, limiting the efficacy of some forms of chemotherapy. The most common chemokine receptor expressed by breast cancer cells is CXCR4, a protein involved in cell migration. CXCR4's ligand Stromal Derived Factor 1 (SDF1-a or CXCL12) is expressed by the tissues to which breast cancer migrates, suggesting that the CXCR4/CXCL12 axis plays a role in metastasis of breast cancer cells to the brain. Endogenously produced endocannabinoids 2-arachidonoylglycerol (2-AG) and anandamide (AEA), and synthetic cannabinoids JWH-015 and Met-F-AEA bind to cannabinoid receptors CB1 and CB2. Cannabinoid receptor inhibition by synthetic cannabinoids has been shown to block CXCR4/CXCL12-mediated *in vitro* migration of immune cells. Due to the high expression of CB1 receptor in the brain, cannabinoids have the ability to cross the blood brain barrier, implicating their capacity to inhibit breast cancer cell metastasis to the brain. Therefore, we explored the ability of endogenous and synthetic cannabinoids to inhibit CXCR4/CXCL12-induced *in vitro* metastatic assays using various breast cancer cell lines such as MDA-MB-231/BR3 (that specifically metastasizes to the brain), NT2.5 (highly metastatic mouse breast cancer cell line), MCF7-CXCR4 (highly expresses CXCR4), and SCP2 (highly metastatic human cell line). These cell lines were used to perform various CXCL12-induced invasive assays such as wound healing, chemotaxis, and chemoinvasion in the presence of endogenous and synthetic cannabinoids. These cannabinoids significantly reduced breast cancer cell chemoinvasion, migration and wound healing. Furthermore, delineation of signaling mechanisms revealed that cannabinoids may inhibit chemoinvasive properties of breast cancer cells by inhibiting CXCL12-induced ERK activity and focal adhesion kinase complex formation. These studies suggest that cannabinoids have the potential to inhibit metastasis of breast cancer cells to various organs including the brain. With future *in vivo* studies using various animal models, including knock-out mouse models which address dosage/targeting issues, endogenous and synthetic cannabinoids could be used to develop new therapies for breast cancer growth and metastasis.

Abbreviations in order of appearance starting in the Introduction:

Δ^9-tetrahydrocannabinol (THC)
anandamide (AEA)
2-arachidonoylglycerol (2-AG)
fatty acid amide hydrolase (FAAH)
monoacylglycerol lipase (MAGL)
(R)-(+)-methanandamide (Met-f-AEA)
G-protein-coupled receptors (GPCRs)
polyoma middle T oncoprotein (PyMT)
extracellular signal-regulated kinases (ERK)
phosphoinositide 3-kinase (PI3K)
p38 mitogen-activated protein kinase (p38MAPK)
protein kinase B (AKT)
cyclin kinase inhibitor (p27/KIP1)
cyclin dependent kinase (cdk)
B cell lymphoma 2 (BCL2)
BCL2-associated X protein (Bax)
cyclic adenosine monophosphate (cAMP)
protein kinase A (PKA)
Transient receptor potential channel V1 (TRPV1)
focal adhesion (FA)
epidermal growth factor (EGF)
extracellular matrix (ECM)
focal adhesion kinase (FAK)
Stromal derived factor-1a (SDF-1a or CXCL12)
guanosine diphosphate (GDP)
guanosine triphosphate (GTP)
adenosine triphosphate (ATP)
extracellular signal-regulated kinase 1 and 2 (ERK1/2 or mitogen-activated kinases (MAPK))
sarcoma tyrosine kinase (src)
nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB)
MAPK extracellular signal regulated kinases (MEK)
related focal adhesion kinase (RAFTK or PYK2)
Tyrosine-protein phosphatase non-receptor type 11 (PTPN11 or SHP2)
casitas B-lineage lymphoma (Cbl)
mitogen activated protein kinase kinase (MEK)
NF-κB kinase (IKK)
NF-κBα (IκBα)
matrix metalloproteinase 2 (MMP2)
tissue inhibitor of metalloproteinases 2 (TIMP2)
urokinase-type plasminogen activator receptor (uPAR)
endothelial progenitor cells (EPCs)
C-terminal domain (CTD)
epithelial to mesenchymal transition (EMT)
c-terminal truncated cytoplasmic tails (CXCR4-ΔCTD)
epidermal growth factor receptor (EGFR)
human epidermal growth factor receptor 2 (HER2/neu or ErbB-2)
transforming growth factor β (TGF-β)
cellular src (c-src)
hypoxia-inducible factor 1, α subunit (HIF1a)

insulin like growth factor-1 receptor (IGF-1R)
estrogen receptor (ER)
tyrosine kinase binding (TKB)
ductal carcinoma in situ (DCIS)
progesterone receptor (PR)
phospho-AKT (pAKT)
von Hippel Lindau (VHL)
Vascular endothelial growth factor (VEGF)
rearranged during transfection/papillary thyroid carcinoma (RET/PTC)
paired box 3 fusion protein-forkhead box protein O1 (PAX3-FKHR)

Abbreviations in Materials and Methods:
Dulbecco's modified Eagle's medium (DMEM)
fetal bovine serum (FBS)
Roswell Park Memorial Institute (RPMI)
phosphate-buffered saline (PBS)
bovine serum albumin (BSA)
fluorescence-activated cell sorting (FACS)
serum-free medium (SFM)
3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrasodium bromide (MTT)
optical density (OD)
radio immuno precipitate assay (RIPA)
Tris-Buffered Saline Tween-20 (TBST)

Introduction

In the US heart disease is the leading cause of death followed closely by cancer[4]. Breast cancer in women is the second leading cause of cancer-related death after lung cancer[3,5]. Metastasis to the brain, bones, lungs, lymph nodes, and liver, not the primary tumor within the breast, leads to death. Therapies that target the signaling pathways of cell movement and growth may inhibit breast cancer metastasis[6]. Cannabinoids have shown promising anti-cancer effects while causing fewer adverse effects than many contemporary chemotherapies such as Trastuzumab and Tamoxifen. These drugs increase the risk of cardiac dysfunction and endometrial cancer, respectively[130,131]. Anti cancer properties of cannabinoids were discovered over 30 years ago with the observation that THC inhibited lung adenocarcinoma cell growth *in vivo*[11]. Non-psychoactive analogues of THC are being studied to evaluate their therapeutic properties in breast cancer.

Cannabinoids fall into three classes: phytocannabinoids, endogenous cannabinoids, and synthetic cannabinoids. Phytocannabinoids are plant-derived substances that include Δ^9-tetrahydrocannabinol (THC) and cannabidiol (all cannabinoids are pictured in Figure 1). Endogenous cannabinoids are produced in our bodies and mediate physiological functions such as immune function, analgesia, the inflammation response, and metabolic, reproductive, and cardiovascular regulation[7,11]. The two best-studied endocannabinoids are anandamide (AEA) and 2-arachidonoylglycerol (2-AG), which are degraded by fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL) respectively[11]. These enzymes can be targeted to inhibit the break down of endocannabinoids, which could be used for therapueutic purposes. Synthetically produced cannabinoids include JWH-133 and JWH-015, and tend to be more potent than endogenous cannabinoids[104]. Due to the instability of AEA, a more stable analogue has been synthesized, (R)-(+)-methanandamide or Met-f-AEA and is commonly used in its place. Synthetic cannabinoids are further divided into non-classical (CP-55,940) and aminoalkylindole (Win55,212-2) subgroups[109]. Cannabinoids mediate their effects through cannabinoid receptors CB1 and CB2, which are heptahelical $G\alpha_i/G\alpha_o$-protein-coupled receptors (GPCRs), which are proteins that span the cellular membrane and act as the mediators between extra- and intracellular signaling transduction components[104,109]. CB1 is primarily located on tissues of the central nervous system and its ligands include Met-f-AEA and other cannabinoids with the similar hydrocarbon tail structures to those of AEA and 2-AG, but with varied head groups[104,112].

The other identified cannabinoid receptor, CB2, resides on immune cells[104]. CB2 receptor ligands include JWH-133, JWH-015, and other similarly structured cannabinoids. Cannabinoids that have affinities for both CB1 and CB2 are AEA, 2-AG, CP55,940, and Win55,212-2[112]. The structures of these compounds are varied, as shown below. Compared to expression patterns in normal tissues, cannabinoid receptors CB1 and CB2 are overexpressed on breast and liver cancer cells[12,105].

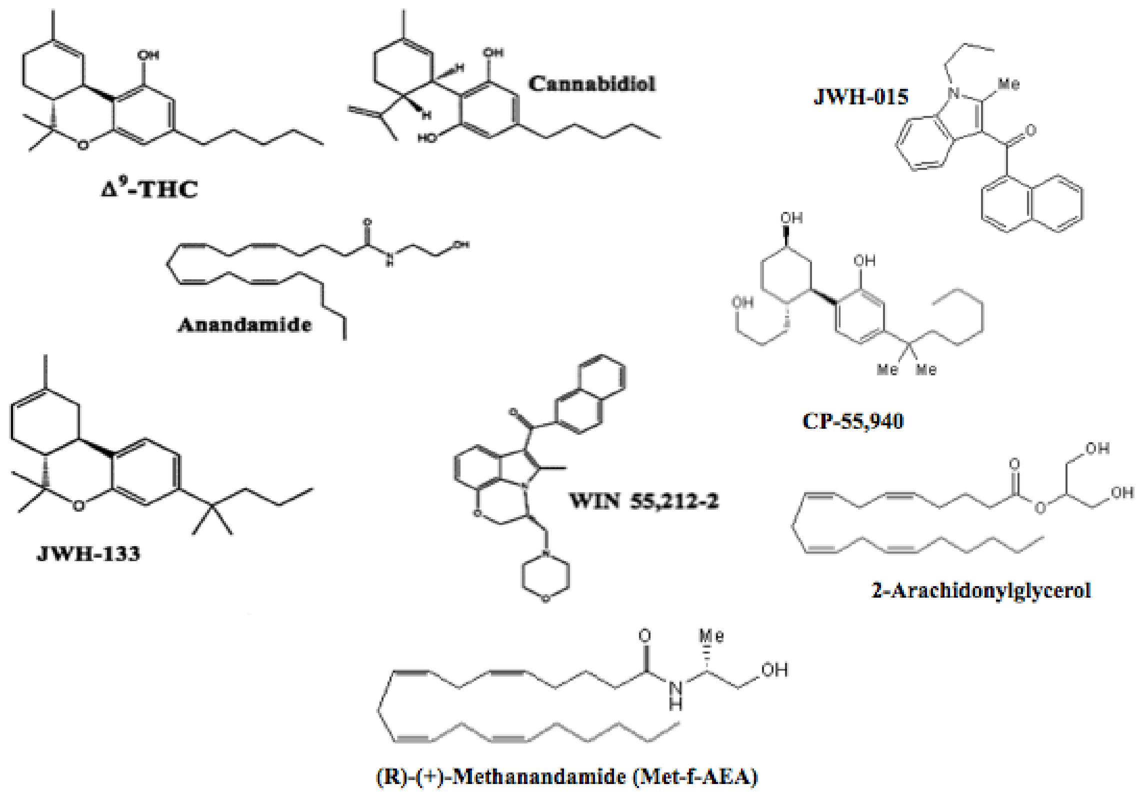


Figure 1. Synthetic, endogenous, and phytocannabinoid structures[103,112].

Cannabinoids such as cannabidiol, JWH-133, and Win55,212-2 inhibit glioma, leukemia, breast, prostate, and colon cancer progression[106,107]. Synthetic cannabinoids have been used to inhibit breast tumor growth *in vivo* using polyoma middle T oncoprotein (PyMT) models[12,109]. Cannabinoids inhibit angiogenesis and arrest the cell cycle, which leads to apoptosis *in vivo*[12,113,114]. Previous *in vitro* and *in vivo* studies indicate that cannabinoids possess both anti- and pro-apoptotic effects, but inhibit migration, metastasis, and invasion[6,11,12,13].

Inhibition of proliferation, which varies depending on cannabinoid dosage and breast cancer cell line treated, is mediated by a variety of well-known protein signaling pathways, including extracellular signal-regulated kinases (ERK), phosphoinositide 3-kinase (PI3K), p38 mitogen-activated protein kinase (p38MAPK), protein kinase B (AKT), and the ceramide pathway[11,12]. These signaling pathways are involved in cell survival, chemotaxis, proliferation, and the tendency of cancer cells to favor aerobic glycolysis over oxidative phosphorylation for energy production[12,22,115]. Cannabinoid binding of CB1 or CB2 causes ceramide synthase to produce lipid molecules of the cell membrane called ceramides, which activates the ERK signaling pathway, leading to cell cycle arrest and apoptosis[11]. ERK stimulation also activates cyclin kinase inhibitor (p27/KIP1), which is involved in cyclin and cyclin dependent kinase (cdk) regulation, leading to induction of apoptosis[116,117,118]. Increased ceramide levels activate p38MAPK, which can stimulate cysteine protease activity or trigger the release of cytochrome c from the mitochondria to cause apoptosis[11]. Increased p53 expression contributes to cell cycle arrest by downregulating B-cell lymphoma 2 (BCL-2), an anti-apoptotic protein and upregulating BCL-2-associated X protein (Bax), a pro-apoptotic protein[11,118]. Modulation of these proteins causes caspase activation, which are cysteine-aspartic proteases responsible for apoptosis and inflammation[118,119]. CB1 and CB2 activation decreases adenylyl cyclase, cyclic adenosine monophosphate (cAMP), and protein kinase A (PKA) activity. Downregulation of these proteins causes decreased gene transcription and induction of apoptosis[116,117,120]. Transient receptor potential channel V1 (TRPV1) activation increases intracellular hydrogen peroxide concentration, calcium levels, and causes cytochrome c dissociation from the mitochondria, also leading to apoptosis (Figure 2)[11,121].

JWH-015 and Win55,212-2 inhibit focal adhesion (FA) formation, which is stimulated by epidermal growth factor (EGF) and integrin clustering and binding[104]. FAs regulate apoptosis, cell migration, and proliferation, and cause signaling proteins to gather in areas where integrins aggregate and bind[123]. Integrins are cell adhesion receptors, which mediate many intracellular signaling pathways and are involved genetic and autoimmune diseases, as well as cancer development[124]. FAs are the primary links between the cell and the extracellular matrix (ECM), formed by focal adhesion kinase (FAK) and vinculin, which connects integrins to the actin cytoskeleton[122,123]. Appropriate regulation of fiber association and disassociation is important for

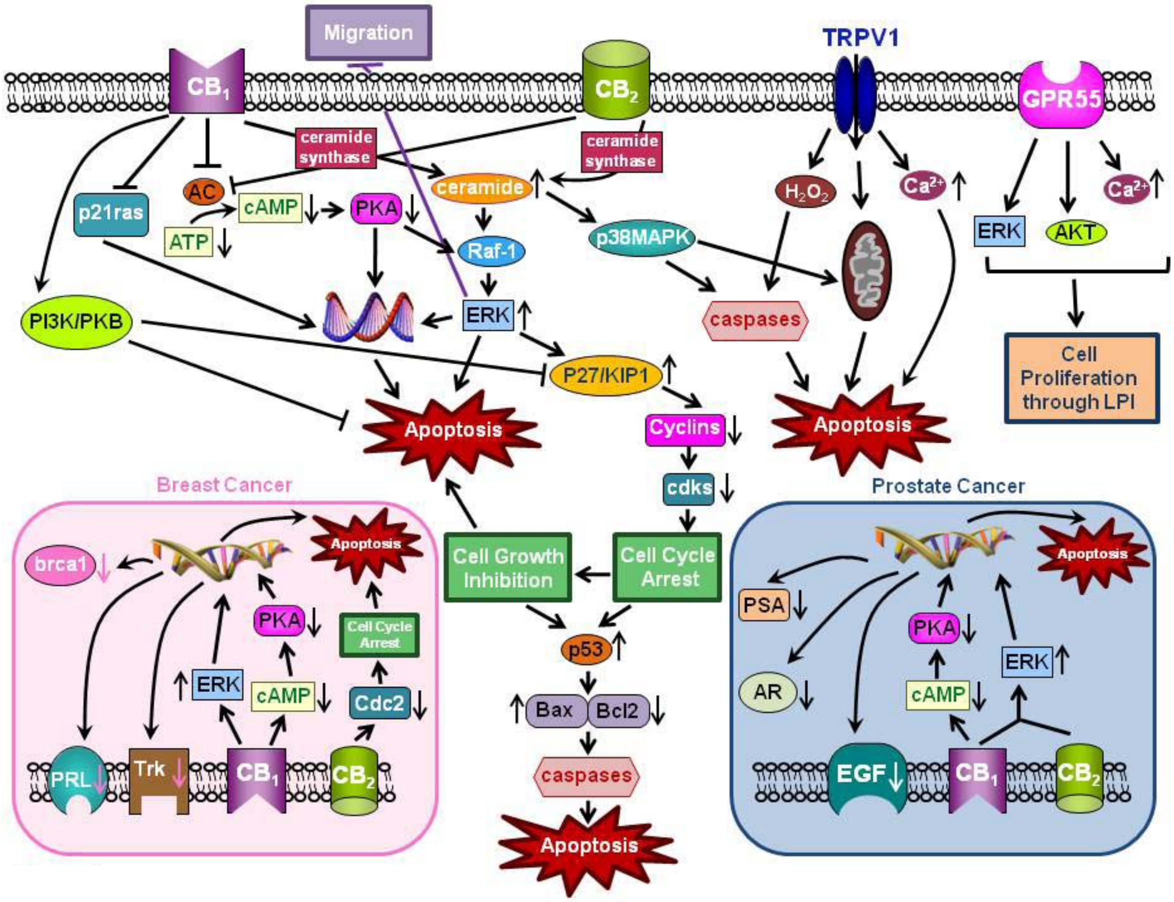


Figure 2. CB1 and CB2 activation can cause apoptosis.

controlling cellular migration and signaling[122]. FAK is responsible for FA turnover and is involved in breast cancer cell invasion and migration[110]. Inhibition of FAK and vinculin causes a significant decrease in normal cell spreading and migration of breast cancer cells[110]. Actin stress fiber formation, also related to focal adhesions, decreases as a result of cannabinoid treatment[104].

The complete mechanism for breast cancer metastasis is little understood, though parts of it are well characterized. Chemokines are a superfamily of small molecular weight signaling proteins around eight to ten kDa that bind GPCRs to promote cell movement[1]. They are responsible in part for hematopoiesis, angiogenesis, targeted immune cell migration to sites of infection, and regulation of cell migration during development[14]. CXCR4 is the most commonly expressed chemokine receptor on breast cancer cells[9], including those used in this study[15]. Metastatic breast cancer tissues have been known to express much higher levels of CXCR4 than normal breast tissues[2,15,16]. Stromal derived factor-1a (SDF-1a or CXCL12) is the chemokine ligand that binds CXCR4 and is synthesized by the areas to which breast cancer metastasizes (Figure 3)[125,126].

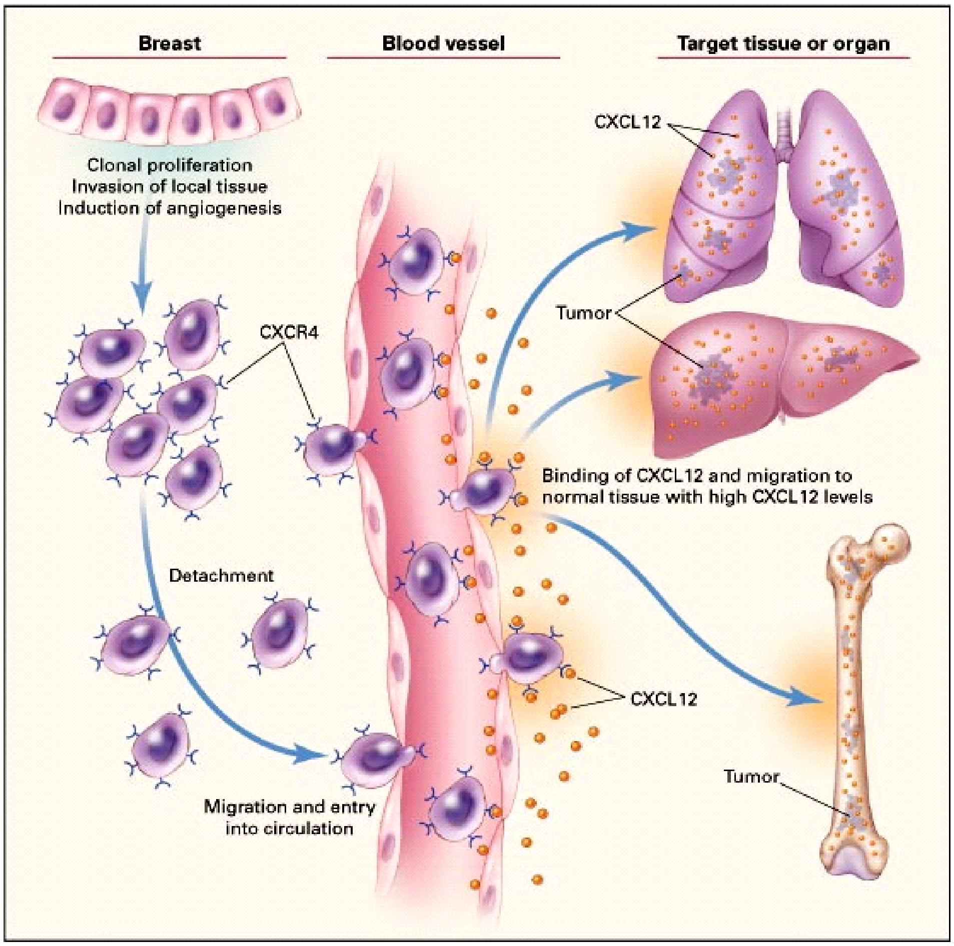


Figure 3. CXCR4/CXCL12-mediated metastasis of breast cancer cells[125,126].

Breast cancer cell migration and metastasis was significantly inhibited when the CXCR4/CXCL12 path was blocked by knocking out CXCL12 production *in vivo*[17]. The CXCR4/CXCL12 axis is known to activate various signaling pathways[34,35,36]. CXCR4 is a GPCR and has been shown to partially mediate its effects through GPCR pathways[37]. These transmembrane proteins bind heterotrimeric G-proteins composed of Gα, Gβ, and Gγ subunits[38,39,40]. In its basal state, CXCR4 is bound to guanosine diphosphate (GDP), but upon binding CXCL12, guanosine triphosphate (GTP) displaces GDP and causes the G-protein to form a βγ dimer and α monomer. The Gα subunit is divided into four subfamilies: Gασ, Gαι, Gαθ, and Gα[12]. CXCR4 mediates its functions primarily through Gαι, which inhibits adenylyl cyclase, an enzyme that converts adenosine triphosphate (ATP) to cAMP[41,42]. This conversion mediates inhibition of extracellular signal-regulated kinase 1 and 2 (ERK1/2 or mitogen-activated kinases (MAPK)), ERK5, and p38MAPK. These proteins are involved in cell proliferation, differentiation, and apoptosis[41]. Gαι mediates CXCR4 signaling through activation of sarcoma tyrosine kinase (src), ERK1/2 and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB)[37,42-45]. The src gene has a tendency to become an oncogene and NF-κB controls DNA transcription and immune response to infection. The ERK pathway is involved in phosphorylation and activation of other cellular proteins and translocation into the nucleus where it phosphorylates and activates transcription factors, leading to changes in gene expression and cell cycle progression[46]. CXCL12-mediated activation of MAPK extracellular signal regulated kinases (MEK) can inhibit apoptosis by inactivating BCL-2[43,47]. The CXCR4/CXCL12 axis may promote cell survival by post-translational inactivation of the cell death machinery and by increased transcription of cell survival-related genes. CXCL12/CXCR4-mediated chemotaxis and proliferation is also mediated by PI3K, which can be activated both by Gβγ and Gα subunits[43,48]. PI3K can then promote tumor cell survival, proliferation and chemotaxis. CXCR4 is known to mediate its effects through protein kinase pathways, such as focal adhesion tyrosine kinases[49,50]. The Ganju group has shown that CXCR4-mediated breast cancer cell motility and invasion is enhanced through activation of FAK and related focal adhesion kinase (RAFTK or PYK2)[51]. CXCR4 has also been shown to activate components of focal adhesion complexes such as Crc and paxilin (Figure 4)[51].

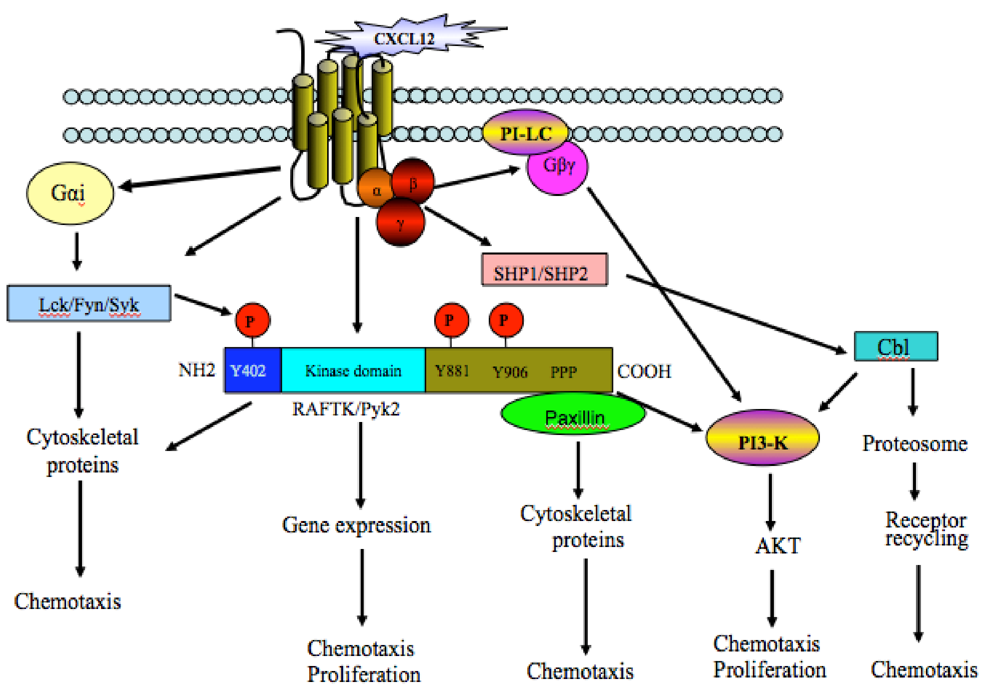


Figure 4. CXCR4/CXCL12 signaling mechanisms that regulate chemotaxis and proliferation in tumor cells[125].

Tyrosine-protein phosphatase non-receptor type 11 (PTPN11 or SHP2) and adaptor-ubiquitin ligases such as casitas B-lineage lymphoma (Cbl) are downstream targets of CXCR4 signaling[37,51-53]. CXCL12 activates PI3K, increasing its association with Cbl and SHP2. Inhibitors of PI3K, RAFTK, and SHP2 significantly inhibit CXCL12-induced chemotaxis and chemo-invasion[37,51,52]. Therefore, CXCL12-induced chemotaxis and chemo-invasion may be mediated through the activation and formation of multimeric-signaling complex with RAFTK, SHP2, and PI3K[37,51,52]. Formation of this complex would lead to cytoskeletal changes and activation of MAP kinases and transcription factors. For instance, CXCL12 treatment of PC-3 cells leads to mitogen activated protein kinase kinase (MEK), NF-κB kinase (IKK) and NF-κBα (IκBα) phosphorylation and nuclear translocation of NF-κB[54]. Activation of these transcription factors enhances expression of metalloproteinases and other proteins, promoting tumorigenesis and cancer progression. Additionally, CXCL12 activates matrix metalloproteinase 2 (MMP2) and MMP9 in breast and prostrate cancer cells[55.] Signaling via the CXCR4 pathway downregulates tissue inhibitor of metalloproteinases 2 (TIMP2) expression, which can increase the invasiveness of prostate cancer cells in the presence of matrix metalloproteinases [56]. Serrati et al. showed that CXCL12 promotes urokinase-type plasminogen activator receptor (uPAR) expression in breast cancer cells with CXCR4,[57] which can induce metastasis *in vivo*[58,59]. CXCL12 also upregulates expression of adhesion molecules such as integrin α4β1 (very late antigen-4 or VLA-4), which can enhance cancer cell invasion[60,61]. The CXCR4/CXCL12 axis enhances β3 integrin expression, leading to the activation of αvβ3 receptors which have been shown to cause prostrate cancer cell adhesion to bone marrow epithelium[62,63]. These signaling pathways are implicated in CXCL12-mediated chemoinvasion and chemotaxis, potentially inducing metastasis (Figure 5).

CXCR4 contains a short C-terminal domain (CTD) with tyrosine residues that are phosphorylated upon ligand binding. CTDs regulate receptor desensitization and down-regulation[64,65]. CXCR4 CTD is essential for receptor regulation and epithelial to mesenchymal transition (EMT). Aberrant CXCR4 function resulting from c-terminal truncated cytoplasmic tails (CXCR4-ΔCTD) occurs in various diseases, including warts, hypogammaglobulinemia, immunodeficiency, and myelokathexis. Breast cancer cells that overexpress CXCR4 with this mutation have altered morphologies, including abnormally high EMT and growth rates[64]. Such is the case for MCF7-CXCR4-ΔCTD, a highly invasive breast cancer cell line, as compared to wild type CXCR4 expressing cells. CXCR4-ΔCTD cells also showed a decrease in E-cadherin and an increase in ERK activation[64]. These studies indicate that CTD region of CXCR4 is important for its regulation, expression and recycling.

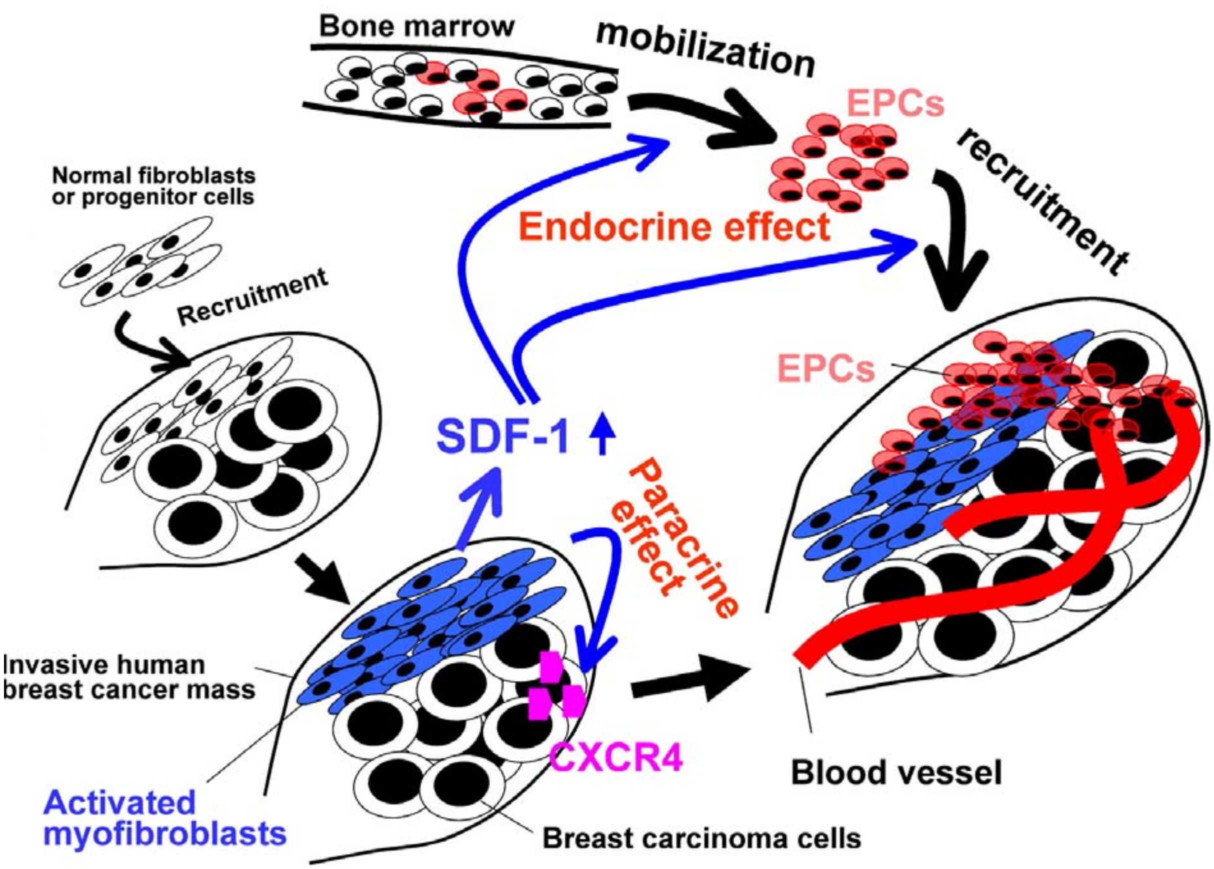


Figure 5. CXCL12 released by stromal fibroblasts promotes tumorigenesis in invasive human breast cancers[127]. Stromal fibroblasts secrete CXCL12 to facilitate tumorigenesis via the **endocrine effect,** in which CXCL12 stimulates angiogenesis by recruiting endothelial progenitor cells (EPCs) to the tumor mass, and by the **paracrine effect**, in which cell survival and tumorigenesis is promoted by direct paracrine stimulation of CXCR4 expressed on the tumor cell surface.

Various factors regulate CXCR4, including p53, which negatively regulates CXCR4 expression in breast cancer cells. Downregulation of wild type p53 has been shown to increase endogenous CXCR4 expression in breast cancer cells and p53 enhancing drugs, PRIMA-1 and CP-31398, reduce expression of CXCR4 at the mRNA and cell-surface level[66]. Activation of p53 also inhibits CXCL12 expression in fibroblasts, modulating adjacent cancer cell migration and invasion[67,68]. Recently, it was shown that wild type p53 reduces CXCL12 expression in stromal fibroblasts[67,68] and stromal fibroblasts that express mutant p53 overexpress CXCL12, enhancing tumor growth in prostrate cancer. Drugs that rescue p53 function may also reduce CXCR4/CXCL12-mediated cell proliferation and metastasis.

The CXCR4 pathway shows crosstalk with other receptors including epidermal growth factor receptor (EGFR), human epidermal growth factor receptor 2 (HER2/neu or ErbB-2) and transforming growth factor β (TGF-β), which regulate tumor growth and metastasis. Activation of CXCR4 enhances ovarian cancer cell EGFR phosphorylation, promoting EGFR trans-activation[69,70]. CXCL12-induced cellular src (c-src) activation may cause this change[69]. CXCL12 has also been shown to activate src kinase, which trans-activates HER2/neu in breast cancer cells[44]. HER2/neu enhances CXCR4 expression, promoting growth and metastasis in lung and breast cancer cells[44,71,72]. EGFR activation in non-small lung cancer cells increases hypoxia-inducible factor 1, α subunit (HIF1α) expression, which increases CXCR4 expression[73]. In the highly invasive and metastatic breast cancer cells MDA-MB-231, CXCR4 formed a complex with insulin like growth factor-1 receptor (IGF-1R), which activates CXCR4 signaling to enhance cell migration and chemotaxis[74]. Crosstalk between CXCR4/CXCL12 and TGF-β1 induces and sustains fibroblast differentiation into myofibroblasts, promoting tumor growth and metastasis in breast cancer cells[75]. Several studies have suggested that there is crosstalk between estrogen receptor (ER) and CXCR4 signaling. For instance, enhanced CXCR4 signaling causes ER-positive breast cancer to become resistant to endocrine therapy[76] and CXCR4 over-expression in ER-positive MCF7 cells enhances hormone independence[77].

Cbl, a 120 kDa protein that contains a tyrosine kinase binding (TKB) domain, becomes phosphorylated at its tyrosine residue in the presence of CXCL12 in breast cancer cells[58]. This protein is an adaptor molecule which can bind to various proteins[78,79] and negatively regulates signaling via ubiquitination and degradation of activated receptor tyrosine kinases[80]. The C-terminal region of Cbl has a proline-rich domain that binds SRC homology 3 (SH3) domain-containing proteins and a group of tyrosine residues that bind SH2 domain-containing proteins[81,82,83]. Some SH3 domain-containing proteins that bind Cbl may also bind components of lipid rafts, thereby mediating chemotaxis[84,85,86]. Cbl regulates cell movement in response to integrin engagement and is involved in the functional organization of the actin cytoskeleton[87,88]. Cbl also interacts with proteins, which co-localize to actin structures and modulate cytoskeletal responses[89]. Cbl deficiency in primary macrophages and osteoclasts has been shown to inhibit cell migration, further suggesting its role in cell migration[90,91,92].

CXCR4 is the most commonly overexpressed chemokine receptor in several human cancers including breast, ovarian, melanoma, and prostrate cancers, among others. Various tissues normally express CXCR4, including bone marrow, blood, spleen, thymus, lymph nodes, pituitary and adrenal glands[1,36]. Immunohistochemical analysis reveals that CXCR4 expression is extremely low or absent in normal breast epithelium, while over 90 % of specimens with atypical ductal hyperplasia tested positive for CXCR4[79,80]. CXCR4 is also present in ductal carcinoma in situ (DCIS) and approximately 75 % of biopsy specimens of invasive ductal carcinoma, meaning CXCR4 expression in these tissues may be a precursor of invasive ductal carcinoma and atypical ductal hyperplasia[57]. High levels of CXCR4 expression has been correlated with decreased overall survival of patients in breast cancer,[80,81] the transition from atypical hyperplasia to invasive cancer,[82] and breast cancer metastasis to the lymph nodes[83]. Poor prognosis in triple negative breast cancer patients, or those whose cancers do not express ER, Her2/neu, or progesterone receptor (PR), is related to high CXCR4 expression[84].

The CXCR4/CXCL12 pathway is involved in several aspects of breast cancer progression including metastasis, the release of cancer cells into the surrounding vasculature or lymphatic system[85,86,87]. Metastatic cells travel to the capillary beds of distant organs where they become embedded to form new masses[86,87]. This process commonly leads to death in breast cancer patients. During metastasis, there are several mechanisms in place to regulate tumor cell trafficking[86,87]. One such pathway is the CXCR4/CXCL12 axis, which mediates organ-specific targeting of metastatic breast cancer cells to tissues that secrete high levels of CXCL12, the lymph nodes, bone, liver, lung, spleen and brain (Figure 4)[90]. Prostate, small cell lung cancer, thyroid, liver, neuroblastoma, and hematological cancers also metastasize to these organs[36,89]. Muller et al. reported that CXCR4 neutralizing antibodies significantly limited metastases to lymph nodes and lung *in vivo*[90]. This observation suggests that the CXCR4/CXCL12 pathway helps regulate metastasis of breast cancer cells. Liang et al. reproduced these results by using siRNAs to block CXCR4 expression at the mRNA level, which decreased breast cancer cell invasion *in vitro* and inhibited metastasis *in vivo*[92]. Additionally, CXCR4 overepxression on cancer cells has been shown to significantly increase the bone metastasis *in vivo*. According to Li et al., HER2/neu expression enhances inhibition of CXCR4 degradation, which would promote breast cancer metastasis[72]. Cancer cells expressing both CXCR4 with EGFR/HER2/neu enhance selective metastases to the bone marrow. Both HER-2 dependent and independent factors elevate phospho-AKT (pAKT) and CXCR4 levels, and activate the HER-2/CXCR4/AKT signaling pathway in primary breast tumors, which may contribute to bone metastasis[94]. The CXCL12/CXCR4 pathway may also be involved in the metastasis of prostrate and breast cancers to the bone[95]. CXCR4/CXCL12 signaling stimulates MMP expression and enhances integrin activity[55,63,96]. Conditions that are known to induce metastasis such as hypoxia have also enhance CXCR4 expression[97]. HIF1, which is normally stimulated by hypoxia, but in many cancers is found to be constitutively active, also increases CXCR4 expression. Inactivating mutated von Hippel Lindau (VHL) tumor suppressor gene, which normally targets HIF1 for degradation, upregulates CXCR4 in adrenal cell carcinomas[98]. Vascular endothelial growth factor (VEGF) and NF-kB activation have the same effect during breast cancer progression and metastasis[45,99]. Oncoproteins such as rearranged during transfection/papillary thyroid carcinoma (RET/PTC) enhance the ability of breast cancer cells to transform by upregulating CXCR4[100]. The paired box 3 fusion protein-forkhead box protein O1 (PAX3-FKHR) also increases CXCR4 expression in rhabdomyosarcoma, stimulating migration and cell adhesion[101]. *In vivo* and *in vitro* neutralization of the CXCR4/CXCL12 signaling leads to a significant inhibition of metastasis[42,59,60]. In prostate and pancreatic cancers, the CXCR4/CXCL12 axis promotes tumor cell transendothelial chemotaxis[45,102]. VEGF, which is involved in angiogenesis and survival of metastatic breast cancer cells, also increases CXCR expression, which promotes their chemotaxis[62]. Breast cancer cells from mammary fat pad xenografts express high levels of cell-surface CXCR4 and show increased CXCL12-induced chemotaxis[64]. Lung metastases have increased CXCR4 expression and migration towards CXCL12[64]. These studies suggest that the CXCR4/CXCL12 pathway is involved in tumor cell trafficking and metastasis regulation to various specific tissues.

Metastatic cells generally have dysfunctional growth regulation mechanisms, undergo cell adhesion alterations, and migrate to distant organs via the blood and lymphatic vessels, leading to secondary tumor formation that represents the most devastating feature of breast and other cancers. Tumor cell motility is one of the most important features of the transition to metastasis[37,38,39]. Molecules that have been shown to enhance tumor cell motility including chemokines are implicated in the development of metastatic lesions[38,39,40,41,42]. Dr. Ganju's research group has shown that non-psychoactive cannabinoids, analogues of THC, inhibit metastasis CXCL12-induced chemotaxis of immune cells[109]. These and previous findings suggest that the CXCR4/CXCL12 pathway is modulated by activation of the cannabinoid system. Thus, we are analyzing the potential of endogenous and synthetic cannabinoids to inhibit CXCL12-mediated migration, invasion, growth, and metastasis by studying cannabinoid-induced protein signaling, and focal adhesion formation in various breast cancer cell lines. We have found promising results from *in vitro* studies using these compounds and are continuing to study the mechanisms of inhibition.

Materials and Methods

Cell Culture

To analyze the effect of cannabinoids on breast cancer, we used various breast cancer cell lines, including human and mouse-derived cells that have high metastatic potential. MDA-MB-231 is a human breast cancer cell line that metastasizes to different organs, where as MDA-MB-231/BR3 is a brain-specific derivative of MDA-MB-231 that has been shown to specifically metastasize to the brain. SCP2 is also a derivative of the MDA-MB-231 cell line that has a high metastatic potential compared to MDA-MB-231. NT2.5 is a mouse-derived mammary cancer cell line also known as MMTV-neu that is highly metastatic. MCF7-CXCR4 is a human breast cancer cell line that overexpresses the CXCR4 receptor.

MDA-MB-231, MDA-MB-231/BR3, NT2.5, and SCP2 cells were cultured in complete medium (Dulbecco's modified Eagle's medium (DMEM), 10% heat inactivated fetal bovine serum (FBS)*, 1% penicillin-streptomycin). MCF7-CXCR4 cells were cultured in complete Roswell Park Memorial Institute (RPMI) medium. Cells were split every 18 – 24 h, depending on the growth rate of the cell line.

*FBS was heated to 60 ^oC for 30 min to inactivate proteins that might interfere with cell culture or any assays that FBS is used in.

Cannabinoid Treatment

Cells were incubated (37 ^oC and 5 % CO_2 humidified environment) in DMEM or RPMI without FBS or penicillin-streptomycin (serum-starved) and incubated with various concentrations of JWH-015, Met-f-AEA, 2-AG, WIN-55,212-2, or ethanol (vehicle) for 4 to 24

h. The CXCR4 ligand CXCL12 (100 ng/mL) was used as a chemoattractant for metastasis and invasion studies, and as a stimulant for signaling studies.

FACS Analysis

Before analyzing the effect cannabinoid treatment on CXCL12-mediated tumor promotion of breast cancer cells, we analyzed the cell surface expression of chemokine receptor CXCR4 and cannabinoid receptor CB2 using fluorescence-activated cell sorting (FACS). CB2 expression was determined because the cannabinoids we used for the other assays in this study are CB2 ligands.

MCF7-CXCR4, SCP2, and NT2.5 cells were washed twice with phosphate-buffered saline (PBS) and blocked (incubated) for 30 min in PBS with 3 % bovine serum albumin (BSA). Cells were then stained using anti-CB2 antibody or anti-CXCR4 antibody for 1 h and washed three times in iced PBS with 3 % BSA. Cells were then incubated for 30 min with fluorescein-labeled secondary antibody in PBS with 3 % BSA before washing three times in the PBS-BSA solution. Cells were transferred into 500 μL PBS and analyzed by flow cytometry.

Transwell Migration Assays

Metastasis and invasion can be modeled *in vitro* by a transwell migration assay. After overnight serum starvation and treatment, cells are loaded into transwell plates and incubated for 6 to 24 h to determine the effects of the drug on metastasis and invasion. A transwell plate is a 24-well plate that has inserts for 12 wells which, when dropped into the wells, create two chambers per well that are separated by a membrane attached to the removable insert. Medium with or without chemoattractant is added to the bottom chamber, and cells are added to the top chamber. After an appropriate amount of time, which depends on the invasive nature of the cells, adherent cells can be fixed to the membrane, stained, photographed, and counted. Suspension cells that have migrated will be floating in the bottom chamber at the end of the assay and can be counted using a hemacytometer.

Cells were treated 12 h in serum-free medium (SFM) containing AEA, 2-AG, or vehicle were allowed to migrate through semi-permeable polycarbonate membranes of transwell migration plates (BD Biosciences). The upper chambers contained 1.5×10^5 cells per well (150 μL of 1×10^6 cells/mL) suspended in SFM and the bottom chambers contained 600 μL SFM with 100 ng/mL CXCL12. Cells adherent to the membrane were stained, photographed (Zeiss), and manually counted at the end of the migration.

Scratch Wound Healing Assay

An important mechanism in the progression of breast cancer is its metastasis to the brain, bone, lung, stromal tissue, lymph nodes, spleen and liver. The CXCR4/CXCL12 pathway appears to be involved promoting breast cancer cell movement to specific tissues[90]. Metastatic breast cancer cells express much higher levels of CXCR4 than do normal mammary cells, and the tissues that secrete CXCL12 are those to which the cancer cells metastasize[2,15,16]. Cannabinoids inhibit breast cancer cell migration *in vivo* and *in vitro* [6,11,12,13] and cannabinoid receptors are expressed on the breast cancer cell surface[12,105]. We investigated the ability of cannabinoids to inhibit CXCL12-induced breast cancer cell metastasis and invasion using a scratch wound healing assay. This experiment simulates an environment in which breast cancer cells have the opportunity to metastasize and invade by closing a wound. After creating a wound, the wound closure in presence or absence of cannabinoids could be compared to evaluate how much cannabinoids inhibited wound healing.

Cells grown in 6-well plates to 100 % confluence were serum starved overnight and scratched with a sterile 200 µL pipet tip to create a wound. Cells were washed with SFM and treated with 10 M synthetic and endogenous cannabinoids in SFM in the absence or presence of 100 ng/mL CXCL12 for up to 24 h. Photographs were taken at the beginning and end of wound healing (Zeiss) and wound closure was quantitated using ImageJ.

Cell Viability

After testing the ability of cannabinoids to block metastasis and invasion, we chose to investigate the cytotoxicity of these compounds on breast cancer cells. Inhibition of metastasis and invasion, simulated by the scratch wound healing and migration assays, could have been caused by a number of possible signaling cascades, including induction of apoptosis. To determine cytotoxicity of these compounds, an MTT assay was performed. MTT or 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrasodium bromide is a clear yellow dye that, once added to viable cells, is metabolized by a mitochondrial enzyme, yielding a dark blue formazan compound. This compound is dissolved to a homogenous mixture by addition of the proprietary color development solution. The optical density (OD) can be measured at 570 nm and used to determine a ratio of viable to non-viable cells[11].

MDA-MB-231 and brain-specific MDA-MB-231/BR3 cells were treated with 1, 5, 10, and 20 µM AEA, 2-AG, or vehicle and plated in 96-well flat-bottom plates. Each concentration was tested in duplicate or triplicate and enough wells were filled to test cytotoxicity over a five day period. To prepare reagents, 10 mL pH 7.4 PBS must me mixed with 1 vial MTT (50 mg MTT/vial). Cells were allowed to incubate in a 37 ^{o}C, humidified, 5 % CO_2 environment for 24 h before "day 1" cells were tested. MTT was added (0.01 mL) to the wells to be tested and allowed to incubate 4 h before adding 0.1 mL color development solution (isopropanol with 0.04 N HCl). Optical density can be read within an hour at a test wavelength of 570 nm and a reference wavelength of 630 nm (Chemicon)[11]. This procedure was repeated over five days.

Cell Stimulation

There are many well-characterized signaling pathways that are altered with the onset and progression of breast cancer. To fully understand the causes and effects of cancer, it is crucial to know all of the pathways involved in cancer pathogenesis. As such, we studied the underlying mechanism of cannabinoid inhibition of invasion, metastasis, and migration, by measuring breast cancer cell protein expression changes in response to cannabinoid treatment and chemokine stimulation. After stimulation, cells were lysed and protein expression was measured by Western blot.

Cells were grown in a monolayer to 70 - 80 % confluence and incubated in SFM containing 10 μM JWH-015 or vehicle. Medium with cannabinoid or control was removed, cells were washed with SFM, and stimulated with 100 ng/mL CXCL12 for 0, 5, 15, 30, and 60 min. Immediately after stimulation, cells were placed on ice, washed twice with ice cold PBS (1X), and either lysed or stored in -80 ^{o}C until lysis.

Protein Isolation and Western Blotting

After stimulation, plates of cells were placed on ice for lysis. Excess media was taken off and 150 μL ice-cold radio immuno precipitate assay (RIPA) lysis buffer* containing phosphatase and nuclease inhibitors was added to each plate. After 5 to 15 min, lysed cells were scraped off of the plate and transferred to Eppendorf tubes on ice. Tubes were rotated for 30 min at 4 ^{o}C, centrifuged at 12,000 RPM at 4 ^{o}C, and the pellet was discarded. Remaining lysate was transferred to new Eppendorf tubes on ice for immediate protein estimation or stored at -20 ^{o}C for later protein estimation.

Protein estimation was done according to modified version of the Microplate Assay Protocol (Bio-Rad)[12]: add 25 μL reagent S to 1 mL reagent A and vortex at RT. Prepare 4 concentrations BSA protein standard (1.52 mg/mL protein) by serial dilution to get 1.52 mg/mL, 0.76 mg/mL, 0.38 mg/mL, and 0.19 mg/mL BSA, and set aside on ice. Pipet 25 µL of the reagent A and S mixture into a sufficient number of wells in a 96 well, flat bottomed plate. Add 5 µL of standards and samples in duplicate to each well. Add 200 µL reagent B to each well and set the plate aside with shaking at RT for 10 to 20 min. Read absorbance of each well at 645 nm, making a standard curve with the standard samples. The volume of each sample to be prepared for resolution on the gel can be estimated by dividing the desired amount of protein (ng) by the value of the optical density.

A final amount of 50 ng protein was prepared according to manufacturer's instructions (Invitrogen)[128]: add the calculated volume for each sample to be resolved on the gel in an Eppendorf tube and add a corresponding amount of Novex® Tricine SDS Sample Buffer (2X) and NuPAGE® Reducing Agent (10X). The total volume should be no more than 60 µL for a gel with 10 wells. Boil samples for 5 min. At this point, samples can be stored in -20 ^{o}C for later use or run immediately. 1X running buffer was prepared from NuPAGE® MES Buffer (10X) and add 500 µL NuPAGE® Antioxidant for every 1 L buffer. Enough buffer was added to the running chamber so that all compartments are connected by liquid. Denatured samples and 8 µL Precision Plus Protein Dual Color Standard (BioRad) were loaded into a pre-cast 4 - 12 % Bis-Tris polyacrylamide gel and run at no more than 180 V until the running dye reached the bottom of the gel cassette. The Bio-Rad Semi-dry Transfer Cell system was used to transfer separated proteins to a nitrocellulose membrane at 16 V for 40 min for 1 blot or 1 h for 2 blots. The membrane was blocked using 5 % non-fat dry milk in Tris-Buffered Saline Tween-20 (TBST) for 30 min. Primary antibodies were incubated overnight at 4 ^{o}C with shaking and secondary antibodies were incubated for 2 h at RT with shaking.

*RIPA buffer is prepared using the following recipe[129]: 150 mM sodium chloride, 1.0% NP-40 or Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS (sodium dodecyl sulphate), and 50 mM Tris, pH 8.0.

Immunofluorescence Microscopy

Focal adhesions (FAs) regulate apoptosis, cell migration, and proliferation[123]. Proper FA turnover time, mediated by FAK and vinculin function, is important in normal cellular migration and signaling[110,122]. Thus, we analyzed the effect of cannabinoids on CXCL12-mediated stress fiber formation. Stress fibers are associated with FAs and are also an indication of altered cellular interactions[104].

MCF7-CXCR4, SCP2, and NT2.5 cells were incubated overnight on tissue culture-treated chamber slides with 20 μm JWH-015 or vehicle in SFM and stimulated for 3 h with 100 ng/mL CXCL12. Cells were washed and fixed using 4 % paraformaldehyde, treated with 0.2 % Triton in PBS to permeabilize, and incubated in 3 % BSA in PBS to block. Cells were then stained green for vinculin. Stress fiber and focal adhesion formation was visualized using a confocal microscope.

Statistical Analysis

Student's two-tailed *t* test was used to compare vehicle and cannabinoid-treated groups. A p-value of less than 0.05 was considered significant. On graphs, * denotes $p < 0.05$, and ** denotes $p < 0.01$ in comparison to vehicle.

Results

Chemokine and cannabinoid receptors are expressed in breast cancer cells

The CXCR4/CXCL12 axis has been implicated in breast cancer cell metastasis. Previous experiments have shown that cannabinoids inhibits of breast cancer cell migration, invasion, proliferation, and metastasis[6,11,12,13]. Before testing the effect of cannabinoids on CXCL12-mediated breast cancer progression, we analyzed the expression of chemokine receptor CXCR4 and cannabinoid receptor CB2 on the surface of MCF7-CXCR4, SCP2, and NT2.5 cells. CB2, and not CB1, expression was confirmed because the cannabinoids used are CB2 agonists with the exception of Met-f-AEA, which was used sparingly.

As shown in figure 6, both CXCR4 and CB2 are expressed on the surface of MCF7-CXCR4, SCP2, and NT2.5 cells.

Cannabinoids inhibit CXCL12-induced migration

THC and non-psychoactive synthetic and endogenous cannabinoids have been implicated in the reduction of breast cancer metastasis. The Ganju group has previously shown that CXCL12 induces chemotaxis/chemoinvasion of breast cancer cells[6]. We analyzed the effect of endogenous and synthetic cannabinoids on CXCL12-mediated chemoinvasive properties of CXCR4 expressing MCF7-CXCR4 and highly invasive breast cancer cell lines SCP2, MDA-MB-231, MDA-MB-231/BR3, and NT2.5.

CXCL12-mediated migration of wild type MCF7-CXCR4 and SCP2 cells was significantly inhibited by 20 μM JWH-015 (Figure 7 A, B). Statistical analysis gave p values of

0.036 and 0.048 for MCF7-CXCR4 and SCP2 cells, respectively. AEA (20 μM) inhibited CXCL12-induced migration of MDA-MB-231 cells by up to 40 % as compared with vehicle-treated cells (Figure 7 C). Concentrations of 20 μM AEA and 2-AG inhibited CXCL12-induced migration of MDA-MB-231 and brain specific MDA-MB-231/BR3 cells by approximately 30 % (Figure 7 D, E, F). Statistical analysis by two-tailed equal variance *t*-test: * $p < 0.05$, and ** $p < 0.01$ in comparison to vehicle. CXCL12-mediated migration of NT2.5 cells was inhibited by 10 μM JWH-015 (data not shown).

These studies suggest that both endogenous and synthetic cannabinoids have the capabilities to inhibit CXCL12-induced migration and chemoinvasion of various breast cancer cell lines.

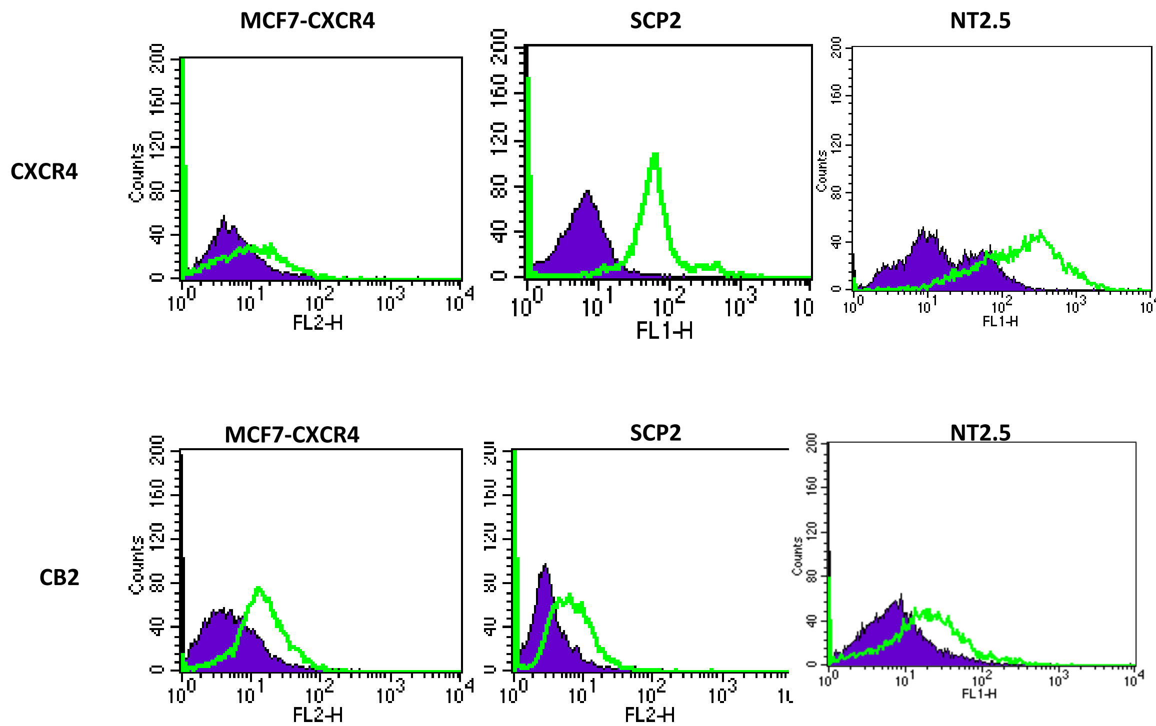


Figure 6: CXCR4 and CB2 are expressed on the surface of MCF7-CXCR4, SCP2, and NT2.5 cells. MCF-7/CXCR4, SCP2 and NT2.5 cells were washed twice with phosphate-buffered saline (PBS) and blocked (incubated) for 30 min in PBS with 3 % bovine serum albumin (BSA). Cells were then stained using anti-CB2 antibody or anti-CXCR4 antibody for 1 h and washed three times in iced PBS with 3 % BSA. Cells were then incubated for 30 min with fluorescein-labeled secondary antibody in PBS with 3 % BSA before washing three times in the PBS-BSA solution. Cells were transferred into 500 mL PBS and analyzed by flow cytometry for surface expression of cannabinoid receptor CB2 and chemokine receptor CXCR4.

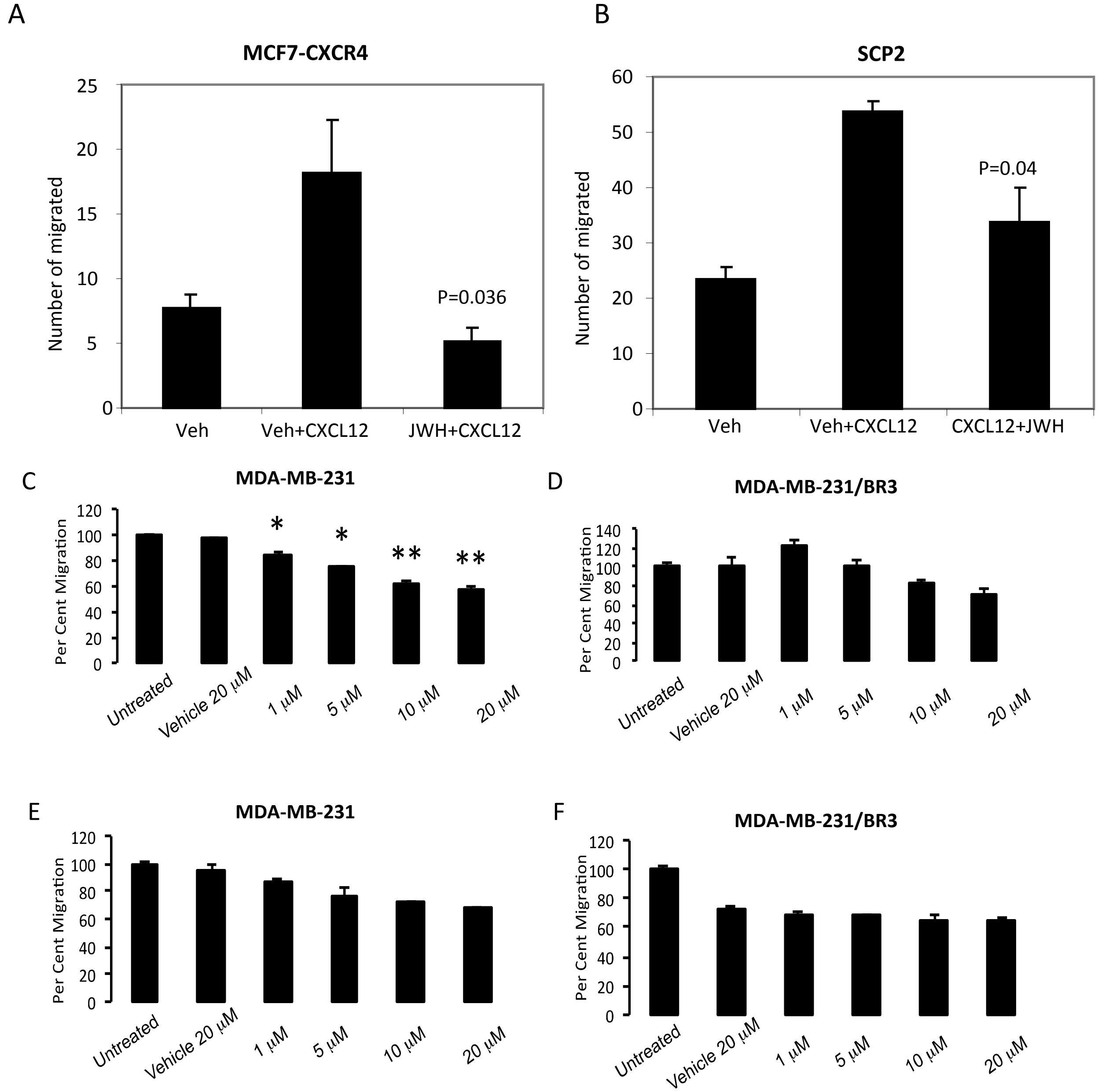


Figure 7. Cannabinoids inhibit CXCL12-mediated migration of breast cancer cells. (A) MCF7-CXCR4 and (B) SCP2 cells were treated overnight with 20 µM JWH-015 or ethanol (vehicle) in serum-free medium (SFM). (C) MDA-MB-231 and (D) MDA-MB-231/BR3 were treated with various concentrations of AEA or vehicle. (E) MDA-MB-231 and (F) MDA-MB-231/BR3 were treated with various concentrations of 2-AG or vehicle. NT2.5 cells were treated with 10 µM JWH-015 or vehicle (data not shown). Cells were allowed to migrate through semi-permeable polycarbonate membranes of transwell migration plates (BD Biosciences). The upper chambers contained 1.5 x 105

cells per well (150 µL of 1x106 cells/mL) suspended in serum-free media and the bottom chambers contained 600 µL serum-free media with or without 100 ng/mL CXCL12. Cells adherent to the membrane were fixed and stained using HEMA stain. Membranes were photographed (Zeiss) and cells were manually counted at the end of the migration. Statistical analysis by two-tailed equal variance *t*-test: * $p < 0.05$, and ** $p < 0.01$ in comparison to vehicle.

Cannabinoids inhibit CXCL12-induced invasive properties

Cannabinoids have been reported to inhibit the migration and wound healing abilities of breast cancer *in vitro* and *in vivo*[12,13,113,114]. The CXCR4/CXCL12 axis appears to induce migration of cancer cells which express CXCR4 to tissues that secrete CXCL12[90,125]. The wound healing assay simulates an environment in which cancer cells can metastasize and invade into the surrounding area. We first evaluated metastasis and invasion of MDA-MB-231 in the absence of CXCL12. Then, we analyzed CXCL12-mediated metastasis and invasion of MCF7-CXCR4, SCP2, and NT2.5 cells.

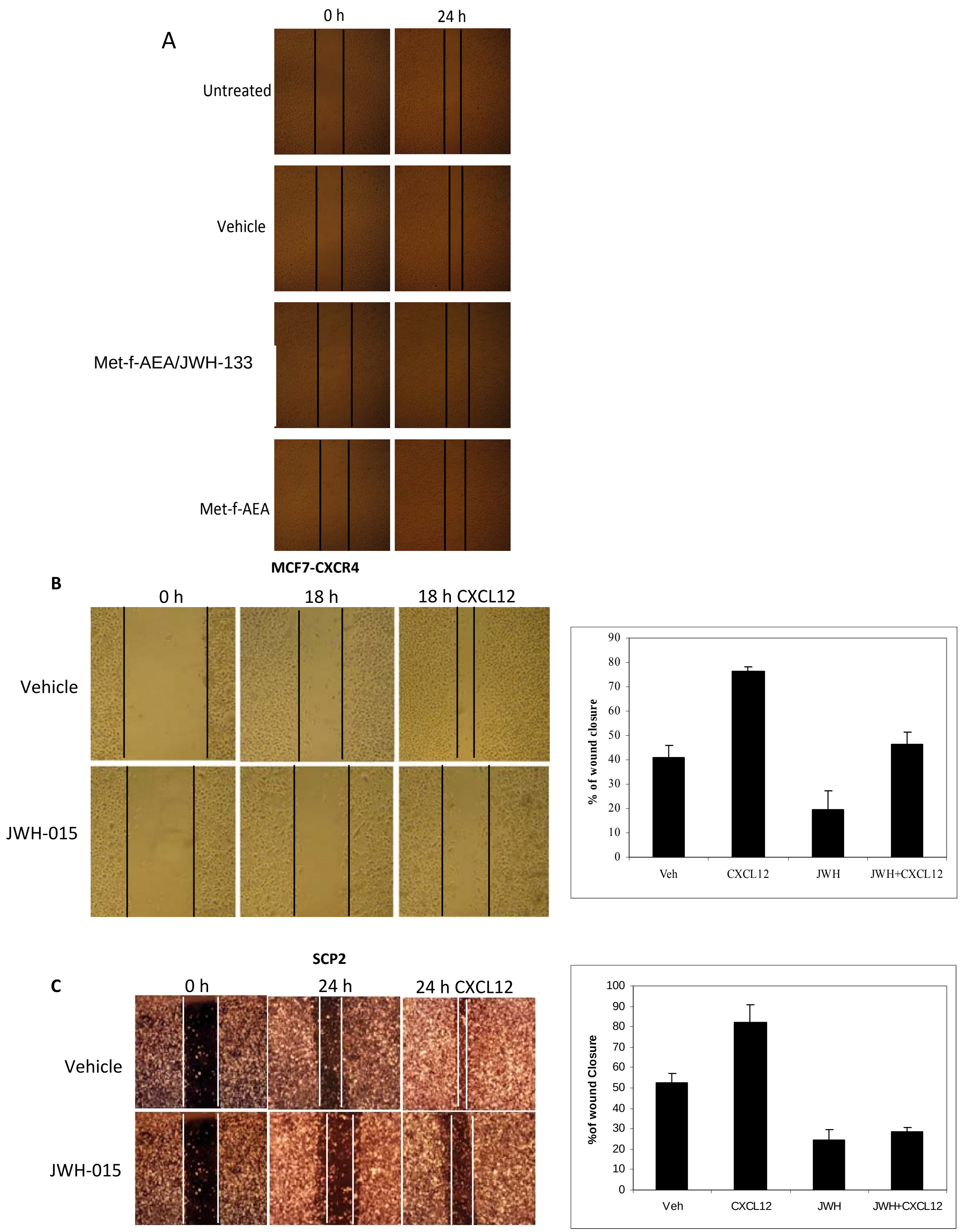
Upon visual inspection of wound closure in Figure 8 (A), it is apparent that 10 µM Met-f-AEA and a combination of 10 µM JWH-133 and Met-f-AEA inhibit wound healing/invasion of highly metastatic MDA-MB-231 cells. CXCL12-mediated wound healing/invasion of MCF7-CXCR4, SCP2, and NT2.5 cells was inhibited by 10 µM JWH-015 (Figure 8 B, C, D).

These studies suggest that synthetic cannabinoids have the capabilities to inhibit CXCL12-induced wound healing/invasive properties of various breast cancer cell lines.

Cannabinoids are not cytotoxic

In some cases, cannabinoids have been found to exert either anti- or pro-apoptotic effects on cancer cells[11,12]. It is important to verify whether inhibition of CXCL12-mediated migration, metastasis, and invasion was caused by cannabinoid crosstalk with the CXCR4/CXCL12 pathway or by cannabinoid cytotoxicity. For this reason, we analyzed proliferation of breast cancer cells treated with cannabinoids over a period of five days using the MTT assay.

Neither AEA nor 2-AG in a concentration of up to 20 µM had cytotoxic effects on MDA-MB-231 and MDA-MB-231/BR3 using the MTT assay (data not shown). OD was measured and no significant induction of apoptosis resulted from endocannabinoid treatment.


A
0 h
24 h
Untreated
Vehicle
Met-f-AEA/JWH-133
Met-f-AEA
MCF7-CXCR4
B
0 h
18 h
18 h CXCL12
Vehicle
JWH-015
% of wound closure
0
10
20
30
40
50
60
70
80
90
Veh
CXCL12
JWH
JWH+CXCL12
SCP2
C
0 h
24 h
24 h CXCL12
Vehicle
JWH-015
%of wound Closure
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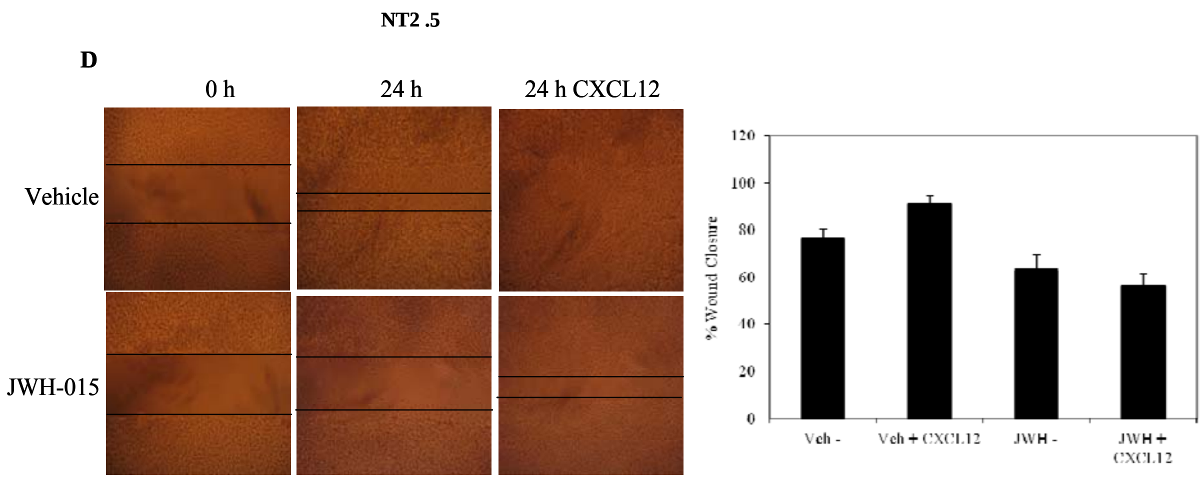

Figure 8. Cannabinoids inhibit CXCL12-mediated wound healing/invasion of breast cancer cells. Cells were grown to 100 % confluence in complete medium in six-well plates and scratched with a 200 mL pipet tip to create a wound. (A) MDA-MB-231 cells were treated overnight with 10 µM JWH-133, a combination of 10 µM JWH-133 and 10 µM Met-f-AEA, or vehicle in SFM. (B) MCF-7/CXCR4-WT, (C) SCP2, and (D) NT2.5 cells were treated with 20 µM JWH-015 or vehicle with or without CXCL12 (100 ng/ml). Photographs (Zeiss) were taken at the beginning and end of wound healing (after 18 or 24 hrs) and wound closure area was quantitated using ImageJ.

JWH-015 modulates CXCL12-induced ERK signaling

CXCL12 has been shown to activate various signaling pathways including ERK pathways. ERK kinases have been shown to be phosphorylated upon activation. In order to determine if cannabinoids inhibit CXCL12-induced migration through the through inhibition of the ERK pathway, we analyzed the effect of cannabinoids on ERK phosphorylation by Western blot using pERK-specific antibodies.

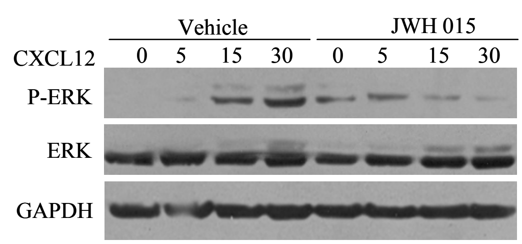

Figure 9: CB2 receptor activation downregulates ERK phosphorylation. SCP2 cells were grown in a monolayer to 70 - 80 % confluence and incubated in SFM containing 10 µM JWH-015 or ethanol. Medium with cannabinoid or control was removed, cells were washed with serum-free medium, and stimulated with 100 ng/mL CXCL12 for 0, 5, 15, 30, and 60 min. Proteins were resolved by Western blot.

As shown in figure 9, JWH-015 inhibited CXCL12-induced phosphorylation of ERK, however there was no effect on ERK total protein content, indicating that cannabinoids specifically inhibit ERK phosphorylation and not protein expression.

Cannabinoids inhibit CXCL12-mediated stress fiber formation in breast cancer cells.

Stress fibers are associated with focal adhesions (FAs), which are links between the actin cytoskeleton and extracellular matrix of cells. These structures help regulate cell migration and proliferation[123]. Focal adhesion kinase (FAK) and vinculin are responsible for correct FA and stress fiber formation, and can be used as markers of altered cellular migration and signaling[110,122]. We analyzed the effect of synthetic cannabinoids on CXCL12-mediated breast cancer stress fiber formation by visualizing vinculin through a confocal microscope.

As shown in figure 10, JWH-015 inhibited stress fiber formation in SCP2 and NT2.5 cells after stimulation with 100 ng/mL CXCL12. Stress fibers are seen in green as a result of anti-vinculin staining and visualization with a confocal microscope.

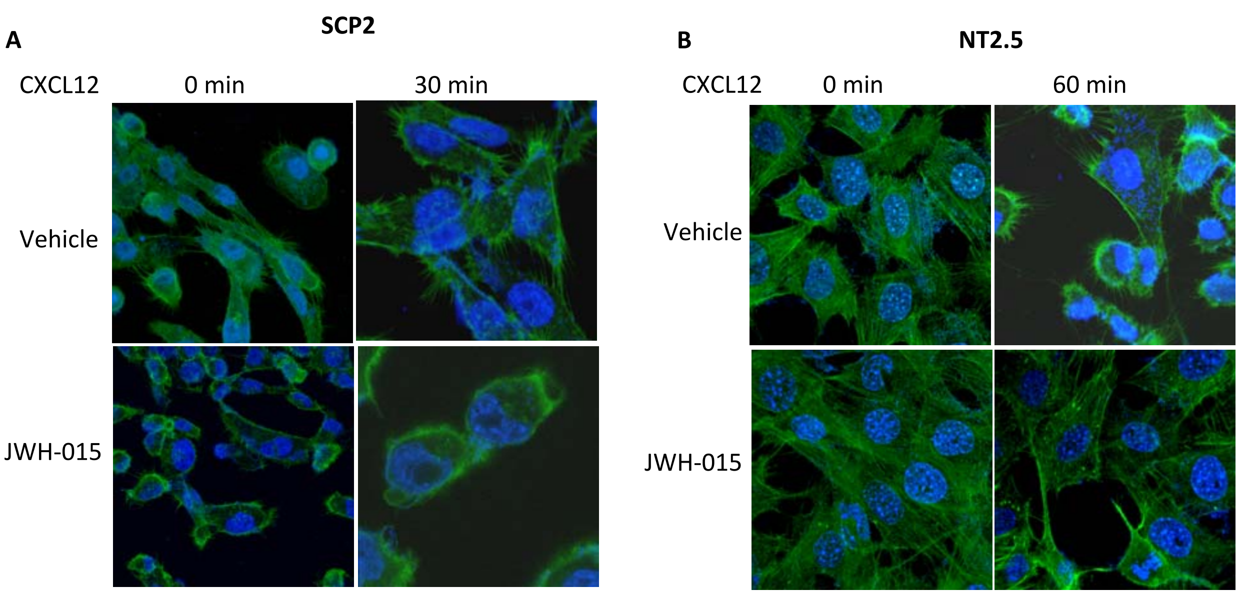

Figure 10. CB2 receptor specific ligand inhibits CXCL12-mediated formation of focal adhesions in breast cancer cells. SCP2 and NT2.5 cells were treated with 20 µm JWH-015 or ethanol overnight and stimulated with CXCL12. Cells were stained for Vinculin (green) and stress fiber and focal adhesion formation was visualized using a confocal microscope.

Discussion

Breast cancer remains a leading cause of death among women worldwide[12]. Breast cancer metastases to the brain, bone, lung, stroma, and liver not the primary tumor in the breast, lead to death[90]. The role of cannabinoids in the treatment of breast cancer is not well known. In previous experiments cannabinoids have shown promising anti-cancer effects. THC has been used *in vivo* to inhibit lung adenocarcinoma growth[11]. JWH-133 and Win55,212-2 have been shown to inhibit glioma, leukemia, breast, prostate, and colon cancer progression[106,107] and breast tumor growth *in vivo* using polyoma middle T oncoprotein (PyMT) models[12,109]. Enzymes that degrade endogenous cannabinoids could be targeted to inhibit breakdown of AEA and 2-AG to exploit their therapeutic potential. Inhibition of FAAH, which breaks down AEA and MAGL, which breaks down 2-AG may have minimal side effects compared to current popular breast cancer treatments including Tamoxifen and Trastuzimab[11]. These drugs are known to increase the risk of endometrial cancer and cardiac dysfunction, respectively[130,131]. Although cannabinoids have been shown to block metastasis, the mechanism is unknown, but could be through the CXCR4/CXCL12 mechanism. In this study, our focus was to understand more about the effects of endogenous and synthetic cannabinoids on CXCL12-mediated invasive properties of various highly metastatic breast cancer cell lines.

The CXCR4/CXCL12 axis appears to mediate cancer cell migration to specific organs and tissues[90,125]. Metastatic breast cancer tissue expresses CXCR4 in much higher levels than normal breast tissue does[2,15,16]. CXCL12, the only known CXCR4 ligand, is secreted by the tissues to which breast cancer metastasizes[90]. *In vivo* CXCL12 knockout models show significantly decreased breast cancer cell migration and metastasis[17]. In our studies, we have confirmed that breast cancer cell lines MCF7-CXCR4, SCP2, and NT2.5 express cannabinoid receptor CB2 and chemokine receptor CXCR4. CB2 expression was verified because the endogenous and synthetic cannabinoids used in this study, with the exception of Met-f-AEA, are CB2 ligands. CXCL12-mediated migration of MCF7-CXCR4 and SCP2 is inhibited by the synthetic cannabinoid JWH-015. Endogenous cannabinoids AEA and 2-AG inhibit CXCL12-mediated breast cancer cell migration of MDA-MB-231 and brain-specific MDA-MB-231/BR3. Additionally, wound healing/invasion of MDA-MB-231 was inhibited by Met-f-AEA and a combination of Met-f-AEA and JWH-133. CXCL12-mediated metastasis and invasion of MCF7-

CXCR4, SCP2, and NT2.5 was inhibited by JWH-015. The concentrations of synthetic and endogenous cannabinoids used to inhibit breast cancer progression *in vitro* were not cytotoxic.

CXCR4/CXCL12 signaling axis has been shown enhances migration of breast cancer through activation of various signaling pathways including ERK kinase[11]. In our studies, we have shown that synthetic cannabinoids inhibit CXCL12-induced activation of ERK by inhibiting phosphorylation of ERK without affecting its total protein content. In addition, we have also shown that cannabinoids may also inhibit stress fiber formation, which have been shown to play an important role in regulating cell migration/invasion/adhesion[104,110]. These properties have been shown to play an important role in metastasis of breast cancer cells[123].

Stress fibers are also associated with focal adhesion (FA) complex formation, which are the primary links between the cellular actin cytoskeleton and the extracellular matrix (ECM)[122,123]. FAK and vinculin are responsible for formation and turnover rate of FAs and are important for the regulation of migration, invasion, and proliferation[104,110,123]. Inhibition of this protein and vinculin causes a significant decrease in normal cell spreading FAK downregulation inhibits migration of breast cancer cells[110]. Function of these proteins can be monitored to evaluate altered cellular behavior. CXCL12-mediated stress fiber formation in SCP2 and NT2.5 cells was inhibited by JWH-015.

Due to the presence of cannabinoid receptors on the brain, cannabinoids have the ability to cross the blood brain barrier, and could potentially be used to inhibit breast cancer metastasis to the brain. Based on this and previous studies, cannabinoids are a desirable addition to current therapies, as they can be endogenously produced and they show promising anti-cancer properties. In future endeavors, more detailed analyses of cannabinoid-mediated effects on CXCL12-induced signaling mechanisms, especially modulation of FAK, RAFTK, PI3K, needs to be carried out. These studies will help us understand the molecular mechanisms underlying cannabinoid-mediated effects on growth and metastasis of breast cancer cells. *In vivo* studies are necessary to validate the results found in this study and to evaluate the effect of cannabinoids and CXCL12 on angiogenesis, tumor formation, and tumor spread. To better determine the clinical possibilities, *in vivo* models addressing cannabinoid receptor tolerance as well as drug dosage and targeting should be explored. These studies represent the beginning stages of a potential addition to current therapies used against breast cancer.

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References

1. Sun X, Cheng G, Hao M, Zheng J, Zhou Z, Zhang J, Taichman RS, Pienta KJ, and Wang J. 2010. CXCL12/CXCR4/CXCR7 chemokine axis and cancer progression. Cancer Metastasis Rev 29:709-722.
2. Bungo F, Ahmed M, Mathias U, Johng RS. 2010. CXCR4 and cancer. Pathology International 60:497-505.
3. Health, United States, 2009 with Special Feature on Medical Technology. U.S. Department of Health and Human Services. Centers for Disease Control and Prevention.
4. Health, United States, 2010 with Special Feature on Medical Technology. U.S. Department of Health and Human Services. Centers for Disease Control and Prevention.
5. Cancer Facts & Figures 2010. American Cancer Society.
6. Fernandis A Z, A Prasad, H Band, R Klösel and R K Ganju. 2004. Regulation of CXCR4-mediated chemotaxis and chemoinvasion of breast cancer cells. Oncogene 23: 157–167
7. Flygare J, Sander B. 2008. The endocannabinoid system in cancer—Potential therapeutic target? Seminars in Cancer Biol 18:176-189.
8. Murphy, PM. 2001. N Engl J Med. 11:833-835.
9. Cronin, PA, Wang, JH, & Redmond, HP. 2010. Hypoxia increases the metastatic ability of breast cancer cells via upregulation of CXCR4. BMC Cancer 10:225.
10. Saghatelian, A et al. McKinney MK, Bandell M, Patapoutian, A, & Cravatt, BF. 2006. A FAAH-Regulated Class of *N*-Acyl Taurines That Activates TRP Ion Channels. Biochem 45(30):9007-9015.
11. Guindon J & Hohmann AG. 2011. The endocannabinoid system and cancer: Therapeutic Implications. British J Pharmacology. In publication.
12. Qamri Z, Preet A, Nasser MW, et al. 2009. Synthetic cannabinoid receptor agonists inhibit tumor growth and metastasis of breast cancer. Molecular Cancer Therapeutics. 8(11)3117-29.
13. Preet A, Ganju RK, Groopman JE. 2008. ^{9}-Tetrahydrocannabinol inhibits epithelial growth factor-induced lung cancer cell migration *in vitro* as well as its growth and metastasis *in vivo*. Oncogene. 27: 339-46.
14. Hinton CV, Avraham S, Avraham HK. 2010. Role of the CXCR4/CXCL12 signaling axis in breast cancer metastasis to the brain. Clin Exp Metastasis 27: 97-105.
15. Tanabe S, Heesen M, Yoshizawa I, Berman MA, Luo Y, Bleul CC et al. 1997. Functional expression of CXC-chemokine recpetor-4/fusin on mouse microglial cells and astrocytes. J Immunology. 159:905-11.
16. Muller A, Homey B, Soto H, Ge N, Catron D, Buchanan ME et al. 2001. Involvement of chemokine receptors in breast cancer metastasis. Nature. 410:50-56.
17. Ma Q, Jones D, Borghesani PR, Segal RA, Nagasawa T, Kishimoto T et al. 1998. Impaired B-lyphopoiesis, myelopoiesis, and derailed cerebellar neuron migration in CXCR4- and SDF-1-deficient mice. Proc Natl Acad Sci USA. 95:9448-53.
18. Ganju RK, Y, Nasser M. CXCL12/CXCR4 Signaling Axis: Role in Tumor Progression and Metastasis. Book chapter *In*: Signaling pathways and molecular

19. Understanding Cancer Series: Cancer
20. Vander Heiden MG, Cantley LC, & Thompson CB. 2009. Understanding the Warburg effect: the metabolic requirements of cell proliferation. Science. 324(5930): 1029-1033
21. Ganju RK et al. 2011. Inhibition Of Cxcr4/Cxcl12-Induced Signaling By Cb2 Receptor Specific Cannabinoid In Breast Cancer. WHERE IS THIS GOING TO BE PUBLISHED???
22. Cairns RA, Harris IS & Mak TW. 2011. Regulation of cancer cell metabolism. Nature Reviews Cancer. 11: 85-95.
23. Warburg O, Wind F, & Negelein E. 1927. The Metabolism Of Tumors In The Body. J General Physiology.
24. Wong KK, Engelman JA, & Cantley LC. 2010. Targeting the PI3K signaling pathway in cancer. Curr Opin Genet Dev. 20(1) 87.
25. Plas DR & Thompson CB. 2005. Akt-dependent transformation: there is more to growth than just surviving. Oncogene. 24, 7435–7442.
26. Khatri S, Yepiskoposyan H, Gallo CA, Tandon P, & Plas DR. 2010. FOXO3a Regulates Glycolysis via Transcriptional Control of Tumor Suppressor TSC1. J Biol Chem. 285(21)15960-5.
27. Fang M, Shen Z, Huang S, Zhao L, Chen S, Mak TW & Wang X. 2010. The ER UDPase ENTPD5 Promotes Protein N-Glycosylation, the Warburg Effect, and Proliferation in the PTEN Pathway. Cell. 143(5)711-24.
28. Semenza GL. 2010. HIF-1: upstream and downstream of cancer metabolism. Current Opinion in Genetics & Development. 20(1)51-6.
29. Dang CV, Kim JW, Gao P & Yustein J. 2008. The interplay between MYC and HIF in cancer. Nature Reviews Cancer. 8 51-6.
30. Shackelford DB & Shaw RJ. 2009. The LKB1–AMPK pathway: metabolism and growth control in tumour suppression. Nature Reviews Cancer. 9:563-75.
31. Mathupala SP, Heese C, & Pedersen PL. 1997. Glucose Catabolism in Cancer Cells: The Type II Hexokinase Promoter Contains Functionally Active Response Elements for the Tumor Suppressor p53. J Biol Chem. 272(36) 22776-80.
32. Matoba S, Kang JG, Patino WD, Wragg A, Boehm M, Gavrilova O, Hurley PJ, Bunz F & Hwang PM. 2006. p53 Regulates Mitochondrial Respiration. Science 312(5780)1650-3.
33. Shakya A, Cooksey R, Cox JE, Wang V, McClain DA & Tantin D. 2009. Oct1 loss of function induces a coordinate metabolic shift that opposes tumorigenicity. Nature Cell Biology. 11:320-7.
34. Otsuka S & Bebb G. 2008. The CXCR4/SDF-1 chemokine receptor axis: a new target therapeutic for non-small cell lung cancer. J Thorac Oncol 2008;3:1379-83.
35. Richmond A. 2010. Chemokine modulation of the tumor microenvironment. Pigment Cell Melanoma Res;23:312-3.
36. Teicher BA & Fricker SP. 2010. CXCL12 (SDF-1)/CXCR4 pathway in cancer. Clin Cancer Res;16:2927-31.
37. Ganju RK, Brubaker SA, Meyer J, et al. The alpha-chemokine, stromal cell-derived factor-1alpha, binds to the transmembrane G-protein-coupled CXCR-4

receptor and activates multiple signal transduction pathways. J Biol Chem 1998;273:23169-75.
38. Balkwill F. Cancer and the chemokine network. Nat Rev Cancer 2004;4:540-50.
39. Lagerstrom MC, Schioth HB. Structural diversity of G protein-coupled receptors and significance for drug discovery. Nat Rev Drug Discov 2008;7:339-57.
40. Viola A, Luster AD. Chemokines and their receptors: drug targets in immunity and inflammation. Annu Rev Pharmacol Toxicol 2008;48:171-97.
41. Goldsmith ZG, Dhanasekaran DN. G protein regulation of MAPK networks. Oncogene 2007;26:3122-42.
42. Mellado M, Rodriguez-Frade JM, Manes S, Martinez AC. Chemokine signaling and functional responses: the role of receptor dimerization and TK pathway activation. Annu Rev Immunol 2001;19:397-421.
43. Bendall LJ, Baraz R, Juarez J, Shen W, Bradstock KF. Defective p38 mitogen-activated protein kinase signaling impairs chemotaxic but not proliferative responses to stromal-derived factor-1alpha in acute lymphoblastic leukemia. Cancer Res 2005;65:3290-8.
44. Cabioglu N, Summy J, Miller C, et al. CXCL-12/stromal cell-derived factor-1alpha transactivates HER2-neu in breast cancer cells by a novel pathway involving Src kinase activation. Cancer Res 2005;65:6493-7.
45. Helbig G, Christopherson KW, 2nd, Bhat-Nakshatri P, et al. NF-kappaB promotes breast cancer cell migration and metastasis by inducing the expression of the chemokine receptor CXCR4. J Biol Chem 2003;278:21631-8.
46. Keshet Y and Seger R. 2010.The MAP Kinase Signaling Cascades: A System of Hundreds of Components Regulates a Diverse Array of Physiological Functions. Methods in Moleuclar Biology. 661(1)3-38.
47. Suzuki Y, Rahman M, Mitsuya H. Diverse transcriptional response of CD4(+) T cells to stromal cell-derived factor (SDF)-1: cell survival promotion and priming effects of SDF-1 on CD4(+) T cells. J Immunol 2001;167:3064-73.
48. Ward SG. T lymphocytes on the move: chemokines, PI 3-kinase and beyond. Trends Immunol 2006;27:80-7.
49. Wang JF, Park IW, Groopman JE. Stromal cell-derived factor-1alpha stimulates tyrosine phosphorylation of multiple focal adhesion proteins and induces migration of hematopoietic progenitor cells: roles of phosphoinositide-3 kinase and protein kinase C. Blood 2000;95:2505-13.
50. Zhang XF, Wang JF, Matczak E, Proper JA, Groopman JE. Janus kinase 2 is involved in stromal cell-derived factor-1alpha-induced tyrosine phosphorylation of focal adhesion proteins and migration of hematopoietic progenitor cells. Blood 2001;97:3342-8.
51. Fernandis AZ, Prasad A, Band H, Klosel R, Ganju RK. Regulation of CXCR4-mediated chemotaxis and chemoinvasion of breast cancer cells. Oncogene 2004;23:157-67.
52. Prasad A, Fernandis AZ, Rao Y, Ganju RK. Slit protein-mediated inhibition of CXCR4-induced chemotactic and chemoinvasive signaling pathways in breast cancer cells. J Biol Chem 2004;279:9115-24.
53. Chernock RD, Cherla RP, Ganju RK. SHP2 and cbl participate in alpha-chemokine receptor CXCR4-mediated signaling pathways. Blood 2001;97:608

54. Kukreja P, Abdel-Mageed AB, Mondal D, Liu K, Agrawal KC. Up-regulation of CXCR4 expression in PC-3 cells by stromal-derived factor-1alpha (CXCL12) increases endothelial adhesion and transendothelial migration: role of MEK/ERK signaling pathway-dependent NF-kappaB activation. Cancer Res 2005;65:9891-8.
55. Chinni SR, Sivalogan S, Dong Z, et al. CXCL12/CXCR4 signaling activates Akt-1 and MMP-9 expression in prostate cancer cells: the role of bone microenvironment-associated CXCL12. Prostate 2006;66:32-48.
56. Wang J, Sun Y, Song W, Nor JE, Wang CY, Taichman RS. Diverse signaling pathways through the SDF-1/CXCR4 chemokine axis in prostate cancer cell lines leads to altered patterns of cytokine secretion and angiogenesis. Cell Signal 2005;17:1578-92.
57. Serrati S, Margheri F, Fibbi G, et al. Endothelial cells and normal breast epithelial cells enhance invasion of breast carcinoma cells by CXCR-4-dependent up-regulation of urokinase-type plasminogen activator receptor (uPAR, CD87) expression. J Pathol 2008;214:545-54.
58. Ahmad A, Kong D, Wang Z, Sarkar SH, Banerjee S, Sarkar FH. Down-regulation of uPA and uPAR by 3,3'-diindolylmethane contributes to the inhibition of cell growth and migration of breast cancer cells. J Cell Biochem 2009;108:916-25.
59. Dass K, Ahmad A, Azmi AS, Sarkar SH, Sarkar FH. Evolving role of uPA/uPAR system in human cancers. Cancer Treat Rev 2008;34:122-36.
60. Ngo HT, Leleu X, Lee J, et al. SDF-1/CXCR4 and VLA-4 interaction regulates homing in Waldenstrom macroglobulinemia. Blood 2008;112:150-8.
61. Sanz-Rodriguez F, Hidalgo A, Teixido J. Chemokine stromal cell-derived factor-1alpha modulates VLA-4 integrin-mediated multiple myeloma cell adhesion to CS-1/fibronectin and VCAM-1. Blood 2001;97:346-51.
62. Engl T, Relja B, Blumenberg C, et al. Prostate tumor CXC-chemokine profile correlates with cell adhesion to endothelium and extracellular matrix. Life Sci 2006;78:1784-93.
63. Engl T, Relja B, Marian D, et al. CXCR4 chemokine receptor mediates prostate tumor cell adhesion through alpha5 and beta3 integrins. Neoplasia 2006;8:290-301.
64. Ueda Y, Neel NF, Schutyser E, Raman D, Richmond A. Deletion of the COOH-terminal domain of CXC chemokine receptor 4 leads to the down-regulation of cell-to-cell contact, enhanced motility and proliferation in breast carcinoma cells. Cancer Res 2006;66:5665-75.
65. Rubin JB. Chemokine signaling in cancer: one hump or two? Semin Cancer Biol 2009;19:116-22.
66. Mehta SA, Christopherson KW, Bhat-Nakshatri P, et al. Negative regulation of chemokine receptor CXCR4 by tumor suppressor p53 in breast cancer cells: implications of p53 mutation or isoform expression on breast cancer cell invasion. Oncogene 2007;26:3329-37.
67. Moskovits N, Kalinkovich A, Bar J, Lapidot T, Oren M. p53 Attenuates cancer cell migration and invasion through repression of SDF-1/CXCL12 expression in stromal fibroblasts. Cancer Res 2006;66:10671-6.
68. Addadi Y, Moskovits N, Granot D, et al. p53 status in stromal fibroblasts modulates tumor growth in an SDF1-dependent manner. Cancer Res;70:9650-8.

69. Porcile C, Bajetto A, Barbieri F, et al. Stromal cell-derived factor-1alpha (SDF-1alpha/CXCL12) stimulates ovarian cancer cell growth through the EGF receptor transactivation. Exp Cell Res 2005;308:241-53.
70. Hernandez L, Smirnova T, Kedrin D, et al. The EGF/CSF-1 paracrine invasion loop can be triggered by heregulin beta1 and CXCL12. Cancer Res 2009;69:3221-7.
71. Cabioglu N, Yazici MS, Arun B, et al. CCR7 and CXCR4 as novel biomarkers predicting axillary lymph node metastasis in T1 breast cancer. Clin Cancer Res 2005;11:5686-93.
72. Li YM, Pan Y, Wei Y, et al. Upregulation of CXCR4 is essential for HER2-mediated tumor metastasis. Cancer Cell 2004;6:459-69.
73. Phillips RJ, Mestas J, Gharaee-Kermani M, et al. Epidermal growth factor and hypoxia-induced expression of CXC chemokine receptor 4 on non-small cell lung cancer cells is regulated by the phosphatidylinositol 3-kinase/PTEN/AKT/mammalian target of rapamycin signaling pathway and activation of hypoxia inducible factor-1alpha. J Biol Chem 2005;280:22473-81.
74. Akekawatchai C, Holland JD, Kochetkova M, Wallace JC, McColl SR. Transactivation of CXCR4 by the insulin-like growth factor-1 receptor (IGF-1R) in human MDA-MB-231 breast cancer epithelial cells. J Biol Chem 2005;280:39701-8.
75. Kojima Y, Acar A, Eaton EN, et al. Autocrine TGF-beta and stromal cell-derived factor-1 (SDF-1) signaling drives the evolution of tumor-promoting mammary stromal myofibroblasts. Proc Natl Acad Sci U S A;107:20009-14.
76. Hall JM, Korach KS. Stromal cell-derived factor 1, a novel target of estrogen receptor action, mediates the mitogenic effects of estradiol in ovarian and breast cancer cells. Mol Endocrinol 2003;17:792-803.
77. Rhodes LV, Short SP, Neel NF, et al. Cytokine Receptor CXCR4 Mediates Estrogen-Independent Tumorigenesis, Metastasis, and Resistance to Endocrine Therapy in Human Breast Cancer. Cancer Res;71:603-13.
78. Sun X, Cheng G, Hao M, et al. CXCL12 / CXCR4 / CXCR7 chemokine axis and cancer progression. Cancer Metastasis Rev;29:709-22.
79. Schmid BC, Rudas M, Rezniczek GA, Leodolter S, Zeillinger R. CXCR4 is expressed in ductal carcinoma in situ of the breast and in atypical ductal hyperplasia. Breast Cancer Res Treat 2004;84:247-50.
80. Luker KE, Luker GD. Functions of CXCL12 and CXCR4 in breast cancer. Cancer Lett 2006;238:30-41.
81. Kato M, Kitayama J, Kazama S, Nagawa H. Expression pattern of CXC chemokine receptor-4 is correlated with lymph node metastasis in human invasive ductal carcinoma. Breast Cancer Res 2003;5:R144-50.
82. Allinen M, Beroukhim R, Cai L, et al. Molecular characterization of the tumor microenvironment in breast cancer. Cancer Cell 2004;6:17-32.
83. Kang H, Watkins G, Douglas-Jones A, Mansel RE, Jiang WG. The elevated level of CXCR4 is correlated with nodal metastasis of human breast cancer. Breast 2005;14:360-7.

84. Chu QD, Panu L, Holm NT, Li BD, Johnson LW, Zhang S. High chemokine receptor CXCR4 level in triple negative breast cancer specimens predicts poor clinical outcome. J Surg Res;159:689-95.
85. Kang Y. Functional genomic analysis of cancer metastasis: biologic insights and clinical implications. Expert Rev Mol Diagn 2005;5:385-95.
86. Chiang AC, Massague J. Molecular basis of metastasis. N Engl J Med 2008;359:2814-23.
87. Yang J, Mani SA, Weinberg RA. Exploring a new twist on tumor metastasis. Cancer Res 2006;66:4549-52.
88. Hinton CV, Avraham S, Avraham HK. Role of the CXCR4/CXCL12 signaling axis in breast cancer metastasis to the brain. Clin Exp Metastasis;27:97-105.
89. Furusato B, Mohamed A, Uhlen M, Rhim JS. CXCR4 and cancer. Pathol Int;60:497-505.
90. Muller A, Homey B, Soto H, et al. Involvement of chemokine receptors in breast cancer metastasis. Nature 2001;410:50-6.
91. Murphy PM. Chemokines and the molecular basis of cancer metastasis. N Engl J Med 2001;345:833-5.
92. Liang Z, Wu H, Reddy S, et al. Blockade of invasion and metastasis of breast cancer cells via targeting CXCR4 with an artificial microRNA. Biochem Biophys Res Commun 2007;363:542-6.
93. Cabioglu N, Sahin AA, Morandi P, et al. Chemokine receptors in advanced breast cancer: differential expression in metastatic disease sites with diagnostic and therapeutic implications. Ann Oncol 2009;20:1013-9.
94. Kim R, Arihiro K, Emi M, Tanabe K, Osaki A. Potential role of HER-2; in primary breast tumor with bone metastasis. Oncol Rep 2006;15:1477-84.
95. Hirbe AC, Morgan EA, Weilbaecher KN. The CXCR4/SDF-1 chemokine axis: a potential therapeutic target for bone metastases? Curr Pharm Des;16:1284-90.
96. Struckmann K, Mertz K, Steu S, et al. pVHL co-ordinately regulates CXCR4/CXCL12 and MMP2/MMP9 expression in human clear-cell renal cell carcinoma. J Pathol 2008;214:464-71.
97. Schioppa T, Uranchimeg B, Saccani A, et al. Regulation of the chemokine receptor CXCR4 by hypoxia. J Exp Med 2003;198:1391-402.
98. Staller P, Sulitkova J, Lisztwan J, Moch H, Oakeley EJ, Krek W. Chemokine receptor CXCR4 downregulated by von Hippel-Lindau tumour suppressor pVHL. Nature 2003;425:307-11.
99. Bachelder RE, Wendt MA, Mercurio AM. Vascular endothelial growth factor promotes breast carcinoma invasion in an autocrine manner by regulating the chemokine receptor CXCR4. Cancer Res 2002;62:7203-6.
100. Castellone MD, Guarino V, De Falco V, et al. Functional expression of the CXCR4 chemokine receptor is induced by RET/PTC oncogenes and is a common event in human papillary thyroid carcinomas. Oncogene 2004;23:5958-67.
101. Tarnowski M, Grymula K, Reca R, et al. Regulation of expression of stromal-derived factor-1 receptors: CXCR4 and CXCR7 in human rhabdomyosarcomas. Mol Cancer Res;8:1-14.

102. Barbero S, Bonavia R, Bajetto A, et al. Stromal cell-derived factor 1alpha stimulates human glioblastoma cell growth through the activation of both extracellular signal-regulated kinases 1/2 and Akt. Cancer Res 2003;63:1969-74.
103. Alexander A, Smith PF, Rosengren RJ. 2009. Cannabinoids in the treatment of cancer. Cancer Letters; 285:6-12.
104. Preet A, Qamri Z, Nasser MW, et al. 2011. Cannabinoid Receptors, CB1 and CB2, as Novel Targets for Inhibition of Non-Small Cell Lung Cancer Growth and Metastasis. Cancer Prev Res;4:65-75.
105. Xu X, Liu Y, Huang S, Liu G, Xie C, Zhou J et al. 2006. Overexpression of cannabinoid receptors CB1 and CB2 correlates with improved prognosis of patients with hepatocellular carcinoma. Cancer Genet Cytogenet;171:31–8.
106. Sarfaraz S, Adhami VM, Syed DN, Afaq F, Mukhtar H. 2008. Cannabinoids for cancer treatment: progress and promise. Cancer Res;68:339–42.
107. Sarfaraz S, Afaq F, Adhami VM, Malik A, Mukhtar H. 2006. Cannabinoid receptor agonist-induced apoptosis of human prostate cancer cells LNCaP proceeds through sustained activation of ERK1/2 leading to G1 cell cycle arrest. J Biol Chem;281:39480–91.
108. Caffarel MM, Andradas C, Mira E, Pérez-Gómez E, Cerutti C, Moreno-Bueno G, et al. 2010. Cannabinoids reduce ErbB2-driven breast cancer progression through Akt inhibition. Mol Cancer;9:196.
109. Ghosh S, Preet A Groopman JE, Ganju RK. 2006. Cannabinoid receptor CB2 modulates the CXCL12/CXCR4-mediated chemotaxis of T lymphocytes. Molecular Immunology;43:2169-79.
110. Keefe T, Chan KT, Cortesio CL, & Huttenlocher A. 2009. FAK alters invadopodia and focal adhesion composition and dynamics to regulate breast cancer invasion. J Cell Biol;185(2)357-70.
111. Fabrizio Montecucco, Fabienne Burger, François Mach, and Sabine Steffens. 2008. CB2 cannabinoid receptor agonist JWH-015 modulates human monocyte migration through defined intracellular signaling pathways. Am J Physiol – Heart. 294(3) H1145-55.
112. Tocris Bioscience Online. Accessed June 2011. http://www.tocris.com/pharmacologicalBrowser.php?ItemId=4983
113. Jia W, Hegde VL, Singh NP, et al. 2006. Δ9-tetrahydrocannabinol-induced apoptosis in Jurkat leukemia T cells is regulated by translocation of Bad to mitochondria. Mol Cancer Res;4:549–62.
114. Kogan NM, Blazquez C, Alvarez L, et al. 2006. A cannabinoid quinone inhibits angiogenesis by targeting vascular endothelial cells. Mol Pharmacol;70:51–9.
115. Ellert-Miklaszewska A, Kaminska B, Konarska L. 2005. Cannabinoids down-regulate PI3K/Akt and Erk signalling pathways and activate proapoptotic function of Bad protein. Cell Signal;17:25–37.
116. Kogan NM. 2005. Cannabinoids and Cancer. *Mini Rev Med Chem* 5: 941-952.
117. Sarfaraz S, Adhami VM, Syed DN, Afaq F, Mukhtar H (2008). Cannabinoids for cancer treatment: progress and promise. *Cancer Res* 68: 339-342.

118. Sarfaraz S, Afaq F, Adhami VM, Malik A, Mukhtar H (2006). Cannabinoid receptor agonist-induced apoptosis of human prostate cancer cells LNCaP proceeds through sustained activation of ERK1/2 leading to G1 cell cycle arrest. *J Biol Chem* 281: 39480-39491.
119. Alnemri ES, Livingston DJ, Nicholson DW, Salvesen G, Thornberry NA, Wong WW & Junying Yuan J. 1996. Human ICE/CED-3 Protease Nomenclature. *Cell* 87 (2): 171.
120. Bifulco M, Malfitano AM, Pisanti S, Laezza C (2008). Endocannabinoids in endocrine and related tumours. *Endocr Relat Cancer* 15: 391-408.
121. Maccarrone M, Lorenzon T, Bari M, Melino G, Finazzi-Agro A. 2000. Anandamide induces apoptosis in human cells via vanilloid receptors. Evidence for a protective role of cannabinoid receptors. *J Biol Chem* 275: 31938-31945.
122. Christopher S. Chen[a], Jose L. Alonso[b, 1], Emanuele Ostuni[c, 2], George M. Whitesides[c] and Donald E. Ingber. 2003. Cell shape provides global control of focal adhesion assembly. Biochemical and Biophysical Research Communications. 307(2)355-61.
123. Sastry SK & Burridge K. 2000. Focal Adhesions: A Nexus for Intracellular Signaling and Cytoskeletal Dynamics. Exp Cell Res;261:25-36.
124. Hynes RO. 2002. Integrins: Bidirectional, ReviewAllosteric Signaling Machine. Cell;110:673-87.
125. Ganju RK, Deol Y, Nasser M. 2011. CXCL12/CXCR4 Signaling Axis: Role in Tumor Progression and Metastasis. Book chapter In: Signaling pathways and molecular mediators in metastasis. Edited by Alessandro Fatitis. Published by Springer Science and Business Media.
126. Murphy PM. 2001. Chemokines and the Molecular Basis of Cancer Metastasis N Engl J Med. 345:833-5.
127. Orimo A, Gupta PB, Sgroi DC, Arenzana-Seisdedos F, Delaunay T, Naeem R, Carey VJ, Richardson AL, & Weinberg RA. 2005. Stromal Fibroblasts Present in Invasive Human Breast Carcinomas Promote Tumor Growth and Angiogenesis through Elevated SDF-1/CXCL12 Secretion. Cell; 121:335-48.
128. Invitrogen Life Technologies Online. Accessed June 2011. http://www.invitrogen.com/site/us/en/home/References/protocols/proteins-expression-isolation-and-analysis/sds-page-protocol/one-dimensional-sds-gel-electrophoresis-of-peptides-and-small-proteins-with-pre-cast-gels.html#buff
129. Abcam. Western Blotting – A Beginner's Guide. www.abcam.com/technical
130. Seidman A, Hudis C, Pierri MK, Shak S, Paton V, Ashby M, Murphy M, Stewart SJ, & Deborah Keefe D. 2002. Cardiac Dysfunction in the Trastuzumab Clinical Trials Experience. J Clin Oncology;20(5)1215-21.
131. Bergman L, Beelen MLR, Gallee MPW, Hollema H, Benraadt J, van Leeuwen FE, and the Comprehensive Cancer Centres' ALERT Group. 2000. Risk and prognosis of endometrial cancer after tamoxifen for breast cancer. The Lancet; 356:881-7.